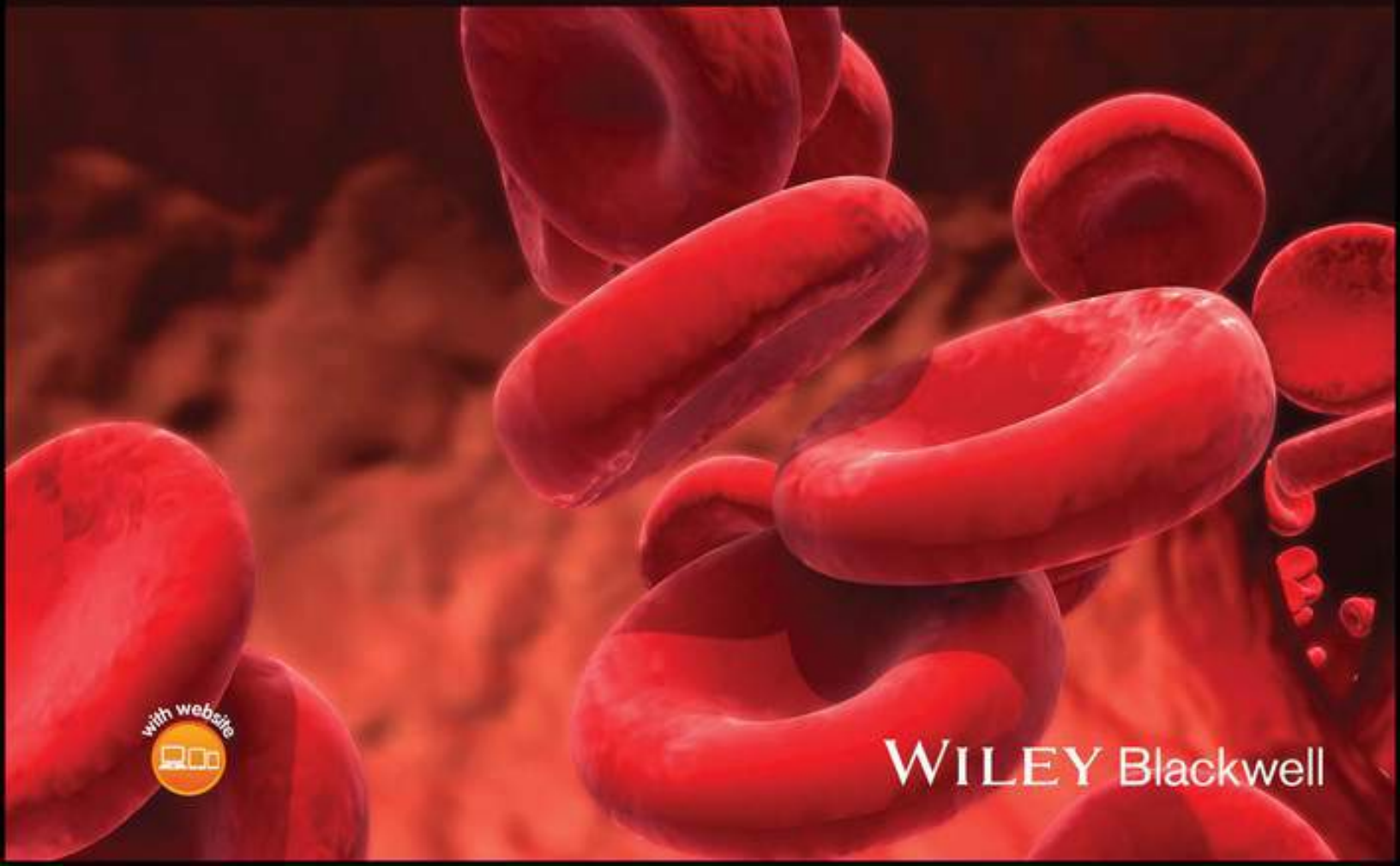
Eighth Edition

Edited by Adam J **Mead** • Michael A **Laffan**
Graham P **Collins** • Deborah **Hay**
Consulting Editor A Victor **Hoffbrand**

Hoffbrand's Postgraduate Haematology



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Hoffbrand's Postgraduate Haematology

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Eighth Edition

WILEY Blackwell

This edition first published 2025
© 2025 by John Wiley & Sons Ltd

Edition History
William Heinemann Ltd (1e, 1972), Butterworth Ltd (2e, 1981; 3e, 1989), Butterworth-Heinmann Ltd (4e, 1999), John Wiley & Sons Ltd (5e, 2005; 6e, 2011; 7e, 2016)

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John Wiley & Sons Ltd, New Era House, 8 Oldlands Way, Bognor Regis, West Sussex, PO22 9NQ, UK

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Library of Congress Cataloging-in-Publication Data
Names: Mead, Adam, editor. | Laffan, Michael A., editor. | Collins, Graham (Hematologist) editor. | Hay, Deborah, 1977- editor. | Hoffbrand, A. V., editor.
Title: Hoffbrand's postgraduate haematology / edited by Adam Mead, Michael Laffan, Graham P. Collins, Deborah Hay ; consulting editor, A. Victor Hoffbrand.
Other titles: Postgraduate haematology
Description: Eighth edition. | Hoboken, NJ : Wiley-Blackwell, 2025. | Preceded by Postgraduate haematology / edited by A. Victor Hoffbrand, Douglas R. Higgs, David Keeling, Atul B. Mehta. Seventh edition. 2016. | Includes bibliographical references and index.
Identifiers: LCCN 2024048152 (print) | LCCN 2024048153 (ebook) | ISBN 9781119706618 (cloth) | ISBN 9781119706663 (adobe pdf) | ISBN 9781119706670 (epub) | ISBN 9781119706687 (obook)
Subjects: MESH: Blood Physiological Phenomena | Hematologic Diseases | Leukemia–blood | Thrombosis–blood
Classification: LCC RC280.H47 (print) | LCC RC280.H47 (ebook) | NLM WH 100 | DDC 616.99/418–dc23/eng/20241206
LC record available at https://lccn.loc.gov/2024048152
LC ebook record available at https://lccn.loc.gov/2024048153

Cover design: Wiley
Cover image: © Design Cells/Adobe Stock Photos

Set in 9.5/12pt MinionPro by Straive, Pondicherry, India

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About the editors

A Victor Hoffbrand qualified in medicine in 1959 at the University of Oxford. After 10 years research at the Royal Postgraduate Medical School, he was appointed in 1974 as professor of Haematology at the Royal Free School of Medicine where he was to spend the rest of his career.

He has published over 700 peer-reviewed scientific papers and chapters in the fields of megaloblastic anaemia, iron chelation, the biochemical, immunological, and molecular biological aspects of leukaemia and lymphoma and stem cell transplantation. He has authored nine editions of *Hoffbrand's Essential Haematology*, five of *The Color Atlas of Clinical Hematology*, four of *Haematology at a Glance* and has edited seven editions of *Postgraduate Haematology*. He authored the haematology section of the *British National Formulary* (1971–1999) and was Chairman of the Editorial Board of the *British Journal of Haematology* from 1990 to 2000. With Robin Foa, he edited *Reviews in Clinical and Experimental Haematology* (2000–2005) and also co-edited with Malcolm Brenner seven editions of *Recent Advances of Haematology*. In 2023, he authored *The Folate Story: A Vitamin Under the Microscope*.

He is a past-president of the British Society for Haematology and was one of the two UK founding councillors of the European Haematology Association. He has lectured in over 40 countries and been visiting professor in Melbourne, Toronto, Rawalpindi, Chandigarh and South Africa. He has advised the Ministries of Health in Kuwait, Cyprus, Hong Kong and Lebanon on their haematology services.

He has been awarded Honorary DSc degrees from Queen Mary (2012) and University College (2019), London. In 2018, he received the Sultan Bin Khalifa International Thalassemia Award, the President's Medal of the Royal College of Physicians and the Wallace H. Coulter Award for Lifetime Achievement, the American Society of Hematology. In 2019, he was awarded the British Society for Haematology Lifetime Achievement Award. He was elected F. Med. Sci. in 2000 and from 2012 to 2013 he was a fellow commoner at The Queen's College, Oxford.

Adam J Mead is a Professor of Haematology in the University of Oxford. Professor Mead is the Clinical Lead for Myeloproliferative Neoplasms (MPN) at Oxford University Hospitals NHS Foundation Trust and has been the Principal or Chief Investigator of over 50 clinical trials in MPN and chronic myeloid leukaemia (CML). He chaired the National Cancer Research Institute MPN subgroup from 2016 to 2022. Professor Mead leads a research group at the MRC Weatherall Institute of Molecular Medicine. The overarching focus of his research group is to discover novel and rational therapeutic strategies to address the current unmet need in persons with MPN, fuelled by a better understanding of the biological principles underlying disease development and progression. He has published over 150 peer-reviewed scientific papers and was elected F. Med. Sci in 2024.

Professor Graham P Collins is an associate professor of Haematology at Oxford University and lead of the Haematology and Lymphoma service at Oxford University Hospitals NHS Foundation Trust. He is deputy chair of the National Cancer Research Institute (NCRI) lymphoma clinical study group, past chair of the Hodgkin and T-cell lymphoma study groups and clinical expert on the National CAR-T Clinical Panel for lymphoma. His academic interests are in Hodgkin and high-grade non-Hodgkin lymphoma, and he is chief investigator on a number of investigator-initiated and commercial clinical trials. He has worked with the National Institute for Health and Care Excellence on guidelines for non-Hodgkin lymphoma and has also co-authored British Society of Haematology guidelines on Hodgkin lymphoma and T-cell lymphoma. Professor Collins is founder and co-course director of the national Lymphoma Management Course and is a supervisor for a number of PhD and MD students. He speaks widely at national and international congresses on a variety of lymphoma-related topics.

Deborah Hay qualified in medicine from the University of Oxford and completed postgraduate training in London, Glasgow and Oxford. After a period of research training in red cell haematology at the Weatherall Institute of Molecular Medicine, she was appointed as the clinical tutor for Laboratory Medicine and Pathology at Oxford University Medical School, and subsequently as deputy director of the graduate-entry medical course. Clinically, her time is divided between consultant practice in diagnostic laboratory haematology and haematopathology in Oxford.

Professor Michael A Laffan is a visiting professor of Haemostasis and Thrombosis at Imperial College London. He was previously consultant haematologist at the Imperial College Healthcare NHS Trust and director of the Hammersmith Hospital Haemophilia Centre. He received his medical degree from the University of Oxford and later trained in Haematology at the Hammersmith and Royal Free Hospitals in London before joining the Royal Postgraduate Medical School, later Imperial College Medical School, as senior lecturer and honorary consultant in 1992.

Professor Laffan is the author of numerous guidelines and contributions to textbooks and is an editor of Practical Haematology. He chaired the British Society for Haematology Task Force on Haemostasis and Thrombosis for six years and the United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) von Willebrand disease working party until 2020. He is currently chair of the BSH Guidelines Executive and has been a senior examiner for the RCPath, chair of the North London Training committee and training programme director.

His research interests have been on the structure–function relationship of von Willebrand factor, with a focus on the role of glycosylation, the role of von Willebrand factor in angiogenesis and the mechanisms and regulation of thrombin generation. He was a lead investigator on the BRIDGE study of genetics in bleeding disorders, precursor to the 100k genomes project and on gene therapy trials for haemophilia.

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Preface to the eighth edition by Adam J Mead

It is a huge honour to take on the role of Editor-in-Chief of *Postgraduate Haematology* for its eighth edition. The book has been an important text in haematology for over five decades, with the fourth edition providing essential reading during my postgraduate exams in haematology 20 years ago. It was a particular pleasure therefore to edit the book alongside Professor Victor Hoffbrand, who edited the first seven editions since the book's inception in 1972. For the eighth edition, we have renamed the book *Hoffbrand's Postgraduate Haematology* in honour of Victor's incredible contribution to the text over so many years. We have also established a new editorial team with a broad range of expertise across different areas in haematology. We have extensively updated the book for the new edition which now includes 57 chapters written by international experts in their respective fields, with chapters divided across 6 themes. Haematology remains at the forefront of the application of advances in molecular biology to the management of human disease, and reflecting this, we have introduced a number of entirely new chapters covering important advances in clinical applications of gene editing and therapy, application of next-generation sequencing in haematology and cancer immunotherapy. Additional new chapters are focussed on clonal haematopoesis, macrophages, dendritic cells and histiocytic disorders, obstetric haematology and consultative haemostasis and thrombosis.

In this digital age, I firmly believe that *Hoffbrand's Postgraduate Haematology* will remain a crucial text for those undergoing postgraduate training in haematology, as well as practising clinicians and scientists working in the field. Faced with the challenge of keeping up to date with an increasingly complex landscape across the wonderfully diverse areas encompassed by clinical and laboratory haematology, we hope that *Hoffbrand's Postgraduate Haematology* will provide a useful resource of authoritative information.

It is an enormous collaborative effort putting together a 57-chapter textbook, and I am immensely grateful to my co-editors, chapter authors and the team at Wiley for their help and patience along the way.

Preface to the eighth edition by A Victor Hoffbrand

Postgraduate Haematology began life 52 years ago as *Haematology: Tutorials in Postgraduate Medicine*. The authors were all members of the Department of Haematology at the Royal Postgraduate Medical School (RPMS), Hammersmith Hospital. John Dacie, Head of the Department, was first invited to edit the book but with other major book writing commitments, *Practical Haematology* and *The Haemolytic Anaemias*, he suggested Mitchell Lewis and me as editors.

The chapters of this first edition were all written by distinguished colleagues in Dacie's Haematology Department including Ted Gordon-Smith (haemolytic and aplastic anaemias), David Galton (leukaemias, myeloma and lymphomas), John Goldman (leucocytes), Leon Szur (myeloproliferative diseases), Bob Pitney (coagulation), and Sheila Worlledge (blood transfusion). They were based on the lectures we gave in the annual Diploma in Clinical Pathology Course at the RPMS.

The book was not intended as a reference to me but as an easily read text for learning about the blood diseases – their pathogenesis, clinical and laboratory features, relevant diagnostic tests, treatment and prognosis. A suggested reading list was given at the end of each chapter but references in the text were avoided. The title *Postgraduate Haematology* was adopted for the second and subsequent editions published by William Heinemann, Butterworth Heinemann, Arnold, Oxford University Press, Blackwell and now Wiley-Blackwell.

With the vast increase in knowledge of blood and its diseases, the book has inevitably increased in size but all editions have followed the same formula. *Postgraduate Haematology* is intended to provide a comprehensive, well-illustrated text, suitable for higher examinations and to the level of consultant haematologist.

Inevitably, a wider range of editors and authors have been needed since the first edition. Edward Tuddenham joined the original two editors to cover the coagulation section for editions four to six; both Daniel Catovsky (editions five and six) and Anthony Green (edition six) were editors for the neoplastic diseases. The editors of the seventh edition included Douglas Higgs, David Keeling and Atul Mehta. The authors, experts in their respective fields, are from around the world, especially from the UK, Europe and North America.

It has been my privilege to edit the first seven editions of *Postgraduate Haematology*. I am delighted to have been replaced as 'editor-in-chief' for the eighth edition by a younger colleague, the talented Oxford haematologist Adam Mead and to welcome his distinguished team of co-editors. They can be just proud of this superb new edition. Finally, I thank the publisher Wiley-Blackwell for adding my name to the book's title.

Victor Hoffbrand, London, 2024

Preface to the first edition

In this book the authors combine an account of the physiological and biochemical basis of haematological processes with descriptions of the clinical and laboratory features and management of blood disorders. Within this framework, each author has dealt with the individual subjects as they thought appropriate. Because this book is intended to provide a foundation for the study of haematology and is not intended to be a reference book, it reflects, to some extent, the views of the individual authors rather than providing comprehensive detail and a full bibliography. For these the reader is referred to the selected reading given at the end of each chapter. It is hoped that the book will prove a particular value to students taking either the primary or the final part of the examination for membership of the Royal College of Pathologists and the Diplomas of Clinical Pathology. It should also prove useful to physicians wishing to gain special knowledge of haematology and to technicians taking the Advanced Diploma in Haematology of the Institute of Medical Laboratory Technology, or the Higher National Certificate in Medical Laboratory subjects.

We acknowledge kind permission from the editors and publishers of the *British Journal of Haematology*, the *Journal of the Royal College of Physicians of London* and the *Quarterly Journal of Medicine* to reproduce Figures 4.1, 4.5, 4.10, 4.11, 4.12, 9.4 and 9.10, the publishers of *Progress in Haematology* for Figure 7.2 and many other publishers who, together with the authors, have been acknowledged in the text. We are particularly grateful to Professor J. V. Dacie for providing material which formed the basis of many of the original illustrations in Chapters 4–8. We are greatly indebted to Mrs T. Charalambos, Mrs J. Cope and Mrs D. Haysome for secretarial assistance and to Mrs P. Schilling and the Department of Medical Illustration for photomicrography, art work and general photography.

Finally, we are grateful for the invaluable help and for bearance we have received from Mr R. Emery and William Heinemann Medical Books.

London, 1972
A. Victor Hoffbrand
Michael Laffan

About the companion website

This book is accompanied by an instructor companion website:

www.wiley.com/go/mead/HPH8e

The website includes the following:

- Bonus chapter 58
- PDFs of the tables
- PowerPoints of the figures

PART I

General principles of haematology

CHAPTER 1

Human stem cells and haemopoiesis

1

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Introduction

The regenerative potential of tissues in the human body has fascinated researchers for many years, perhaps sparked by the ancient Greek myth describing Prometheus's fate, punished by Zeus to have a vulture feed off his liver during the day, only for the liver to be regenerated and the process repeated daily. Although the ancient Greek myth at that time was unlikely to be based on knowledge on the regenerative potential of the liver, we now know that most tissues in the human body contain regenerative potential to replace cells lost due to their normal limited lifespan, stress, disease or injuries, and that this process depends on tissue-specific stem cells. Of these, the stem cells responsible for the production and maintenance of mature blood cells, the haemopoietic stem cell, remains the most well characterised stem cell system to date. The routine clinical use of bone marrow transplantation to rescue diseased haemopoiesis highlights the impressive power of stem cell therapy, facilitated by the culmination of decades of biological and clinical research. Although representing the only curable treatment option for several disease conditions, it still is not possible to offer this treatment to all patients in need. As such, more research towards both the biology and clinical application of human haemopoietic stem cells are required to further improve our understanding and clinical application of haemopoietic stem cell therapies.

Human haemopoietic stem cells and the haemopoietic differentiation hierarchy

Blood is one of the most regenerative tissues in the body, where every minute millions of mature blood cells need to be replaced in a normal healthy individual due to their finite lifespan. In contrast to solid tissues, blood cells are dispersed throughout the body. Although the majority of mature blood cells are found in peripheral blood and haemopoietic organs such as the bone marrow, spleen, thymus and lymph nodes, mature blood cell infiltration in other organs and tissues are also important for prevention of infections and response to injury. With the exception of long-lived memory B and T cells, and some tissue-resident macrophages, mature blood cells have a short lifespan, ranging from just a few days for mature granulocytes, monocytes and platelets to 120 days for red blood cells (Figure 1.1). Ensuring the continuous replacement of lost cells is critical to maintain normal homeostasis, as insufficient blood cell production results in cytopenia and increased risk for infection, and if not corrected threatens the ability to sustain life. The process of blood cell production, haemopoiesis, results in the daily generation of 10^{11}–10^{12} mature blood cells in an adult healthy individual, numbers which can increase in response to acute stress such as large blood loss, infection or disease conditions.

Haemopoiesis is a tightly regulated cellular process that depend on the lifelong pool of haemopoietic stem cells (HSCs) defined by their ability to self-renew and potential to generate

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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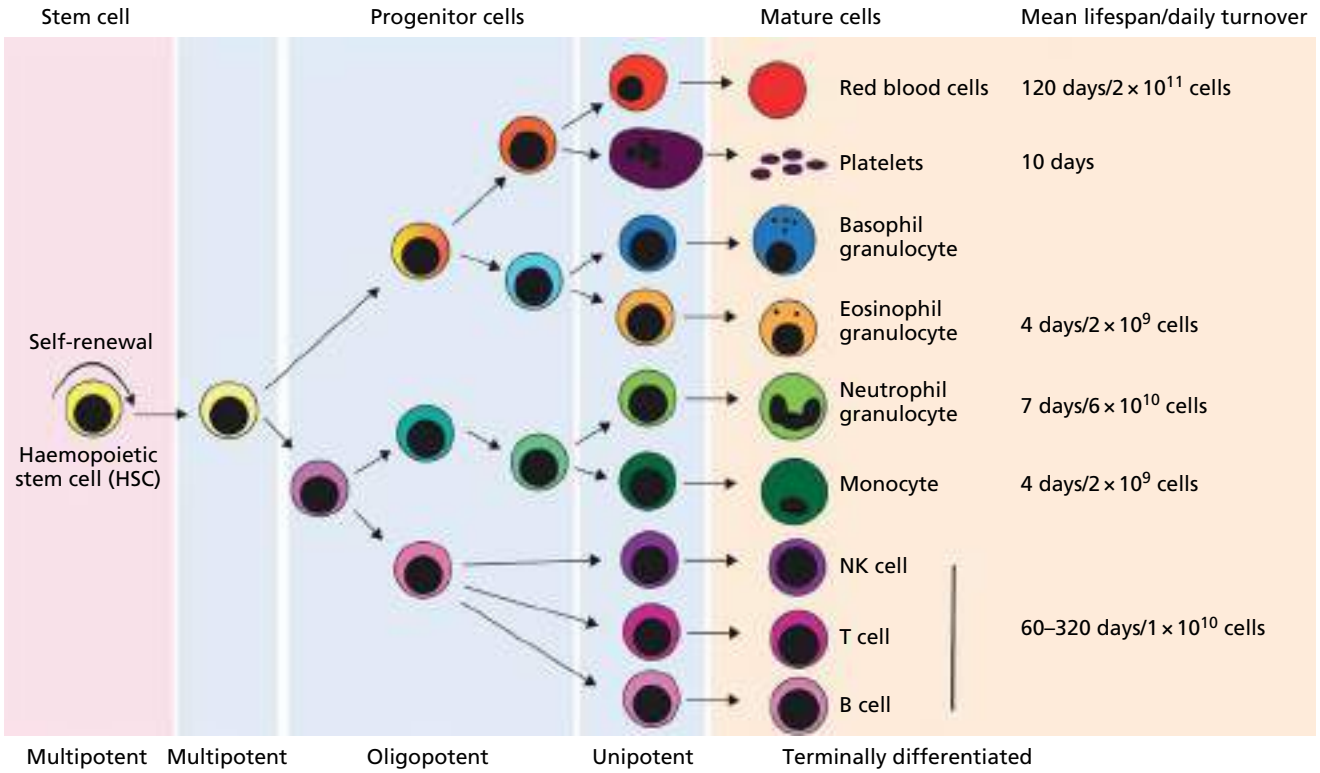


Figure 1.1 The human haemopoietic hierarchy. The human haemopoietic hierarchy has a unidirectional differentiation path (arrows going from left to right only) with the haemopoietic stem cell at the apex and the mature blood cells at the end. Due to the short lifespan and daily turnover of mature blood cells, continuous replenishment of lost cells is facilitated by haemopoietic stem cells through the generation of series of haemopoietic progenitor cells which gradually lose lineage potential. This process of active haemopoiesis is dependent on a limited pool of quiescent and long-lived haemopoietic stem cells in the bone marrow with life-long self-renewal potential and potential to generate all mature blood cells.

all blood cell lineages (multipotent). In adults, this process takes place in the bone marrow, the major site of residency for HSCs after birth. HSCs represent an infrequent population of cells in the bone marrow, and studies to understand how such a rare population of cells can generate trillions of cells on a daily basis has been at the centre of research since their existence was proposed. Studies in model organisms and on material from human subjects have demonstrated that the impressive production of blood cells is achieved through a unidirectional cellular hierarchy where HSCs are located at the apex and give rise to the mature blood cells through the generation of series of intermediate progenitor cell stages. In contrast to HSCs, the majority of these intermediate progenitor cells are short-lived as they lack self-renewal potential and therefore depend on continuous replenishment from the HSCs. The generated progenitor cells are highly proliferative and as they differentiate through the haemopoietic hierarchy, they gradually lose potential to generate one or more lineages. Although the cellular differentiation pathway from human HSCs is still under investigation, studies have suggested that HSCs first give rise to progenitor cells that carry the same potential as HSCs (multipotent) but have lost or reduced ability for self-renewal and therefore will not be sustained for an extended period of time. The multipotent progenitor then gives rise to oligo-potent progenitors that has lost potential for one or more blood cell lineages, which ultimately gives rise to unipotent progenitors restricted towards one blood cell lineage. In both mouse and human bone marrow, many of these intermediate progenitor cell stages have been identified suggesting that the first lineage-restriction that occurs after a multipotent progenitor cell results in division between progenitors with potential to generate lymphoid cells (B, T and NK cells), neutrophils and monocytes/macrophages and progenitors with potential to generate erythroid, megakaryocyte/platelets, eosinophils and basophils. Identification and characterisation of these stages is important not just for understanding the underlying cell biology, but also since these intermediate stages often are perturbed in diseases and haematologic malignancies, they remain relevant for understanding how haemopoietic dysregulation leads to clinical

disease. Although the haemopoietic hierarchy often is displayed as discrete stages of cellular intermediates (Figure 1.1), it is probable that a more gradual continuum of lineage restriction exists, rather than the binary distinct stages typically displayed in figures in the literature.

Human haemopoietic stem cell characteristics

HSCs, similar to stem cells responsible for the regeneration of other tissues, are defined functionally by their ability to self-renew, meaning that they can persist for the duration of life through generation of daughter HSCs, and their capacity for multipotent differentiation, meaning they have the potential to generate all mature haemopoietic cell types. In light of the dependence of blood production on HSCs it is critical that the number of HSCs and their activity is tightly controlled, both under steady-state conditions and under stress conditions. In response to signals from HSCs themselves (intrinsic) and the surrounding environment (extrinsic), HSCs can undergo different fates that regulate the number of HSCs in the body (Figure 1.2). In response to proliferative signals HSCs undergo cell division, where an asymmetric division generates two daughter cells, one which is an exact HSC copy of the parental HSC, endowed with both self-renewal and multipotency, and a differentiated progeny which has lost self-renewal. Such a cell division would result in neither gain or loss of HSCs and therefore preserve the number of HSCs in the bone marrow. Alternatively, HSCs can undergo two different types of symmetric cell divisions. If a HSC undergo a symmetrical self-renewal division, two new daughter HSCs will be generated from the parental HSC, both with self-renewal and multi-potency, resulting in an expansion of HSC numbers. However, if a parental HSC undergo a symmetric differentiation division, two differentiated daughter cells without self-renewal will be generated, resulting in HSC loss. In addition to cell division, HSCs can also undergo apoptosis which similar to symmetric differentiation division results in HSC loss.

Although cell division is required for the progeny generation, cell divisions are infrequently performed by HSCs during steady-state conditions. Instead, most HSCs remain dormant in a quiescent state, referred to as G_0 in the cell cycle. Quiescence is a hallmark feature of HSCs in steady-state conditions, defined

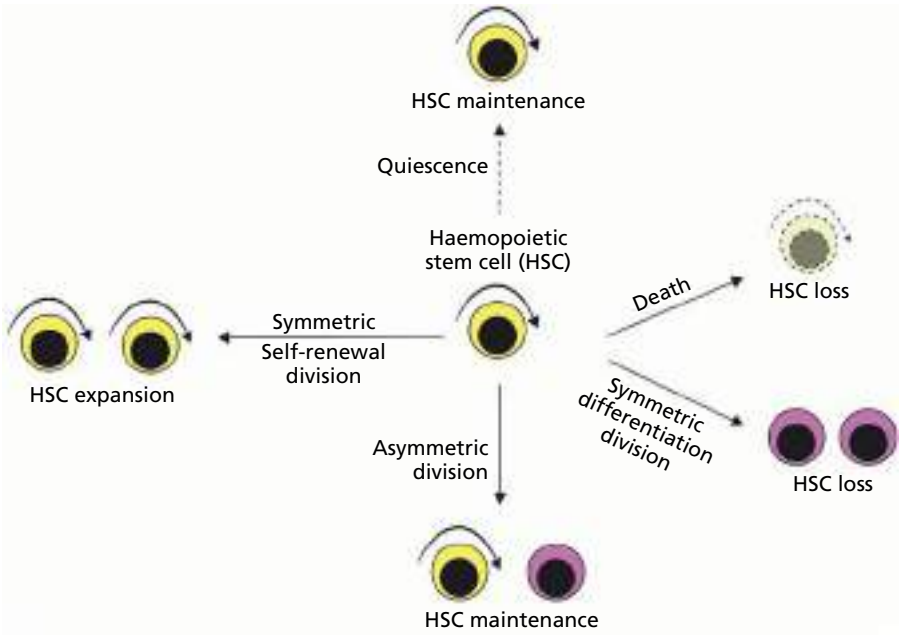


Figure 1.2 Haemopoietic stem cell fate options. Controlling the number of haemopoietic stem cells is critical for preserving normal blood cell production. During steady-state, the majority of haemopoietic stem cells are highly quiescent, protecting the haemopoietic stem cells from the potential harmful effects actively dividing cells are exposed to and preserving the haemopoietic stem cell pool. Upon stimulation, haemopoietic stem cells can be activated and undergo cell division. Symmetric division can either result in haemopoietic stem cell expansion or loss, dependent on the generated progeny being haemopoietic stem cells or differentiated progenitor cells. Asymmetric division, where cell division generated one daughter haemopoietic stem cell and one differentiated progenitor cell, preserve the haemopoietic stem cell pool. Upon exposure to harmful effects, lack of appropriate stimulation or haemopoietic stem cell exhaustion, haemopoietic stem cells can undergo programmed cell death. The fate of haemopoietic stem cells is regulated by the complex interplay between extrinsic (environmental) and intrinsic (inside haemopoietic stem cells) factors.

by infrequent cell cycle divisions and low metabolic activity. This is a critical cellular mechanisms that maintain HSC functional integrity by protecting against HSC exhaustion, harmful substances and DNA damage that can result in leukemic transformation. Quiescence is therefore a critical mechanism for preserving the HSC pool. In light of the high degree of cellular dormancy associated with HSCs, it is proposed that primitive multipotent progenitor cells to a large degree actively contribute to steady-state haemopoietic. Although these progenitors depend on generation from HSCs, they can sustain blood production for long but finite periods of time, reducing the need for a continuously active HSC pool. However, under stress-conditions, also the HSCs are activated and proliferate until normal blood production is restored.

Assays for human haemopoiesis

The existence of HSCs was already proposed in the early 1900s where a common progenitor was proposed to explain the heterogeneity among different blood cells. However, the experimental proof for HSCs was not established until after the post-World War II era when the impact of large-dose irradiation was explored and the lethal effect of irradiation was found to be rescued by bone marrow transplantation. In part, this led to the clinical use of haemopoietic stem cell transplantation which is widely used in the clinic today, but also sparked the development of experimental assays that led to the identification of HSCs. These were spearheaded by two Canadian researchers, James Till and Ernest McCulloch, who first developed an assay that demonstrated the existence of cells with ability to self-renew and produce cells of different blood lineages. These initial studies were performed in mice which after receiving a lethal dose of irradiation were transplanted with bone marrow harvested from a non-irradiated mouse. Upon termination of the mice following transplantation, they noticed distinct nodules in the spleen resembling colonies where an individual colony was demonstrated to originate from a single cell (clonal), contain progeny of multiple blood cell lineages (multipotent) and could be serially transplanted into new irradiated recipients (self-renew). Cells that contained this potential were referred to as colony forming unit (CFU) spleen and formed the birth of stem cell biology and development of additional assays that allowed for the identification and characterisation of HSCs in mouse and man.

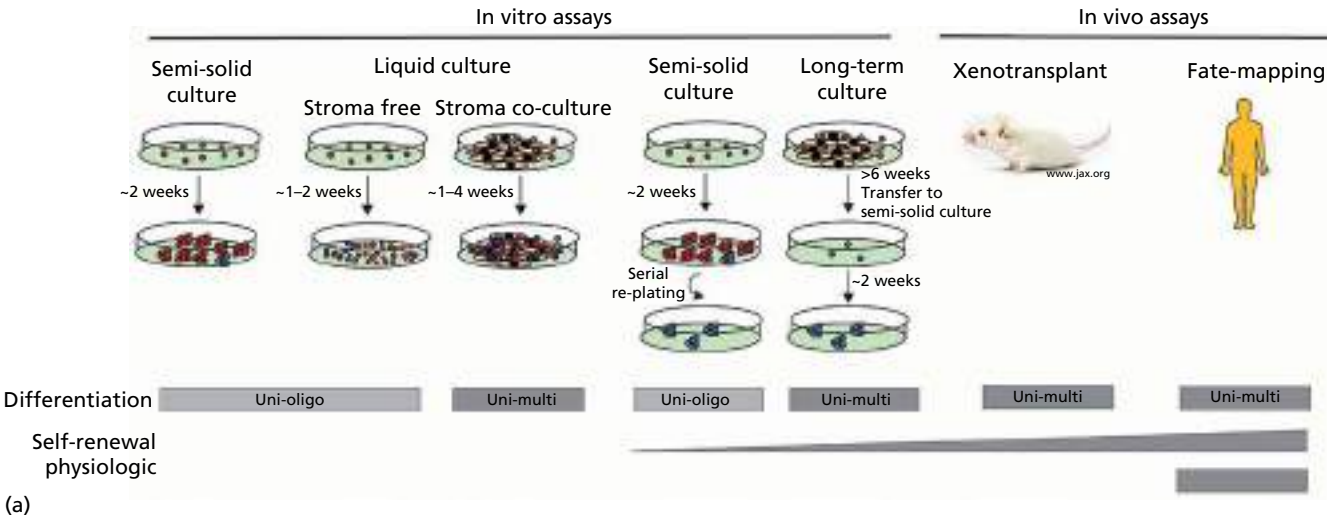
Short-term in vitro assays

In vitro assays (Figure 1.3a) have been instrumental for both identification of distinct haemopoietic stem and progenitor cell populations and the cytokines, as well as other growth factors, that regulate their ability to generate different mature blood cell lineages. Culture of haemopoietic cells in a semi-solid medium composed of methylcellulose supplemented with haemopoietic growth factors supports the proliferation and subsequent differentiation of haemopoietic stem or progenitor cells following short-term culture. Similar to the colonies observed in the spleen (CFU-spleen) of irradiated mice following transplantation of non-irradiated bone marrow, as the proliferation from stem and progenitor cells are physically restricted within the semisolid medium this results in the generation of multiple distinct colonies, where each colony originates from one single stem or progenitor cell. The characteristic colonies can be both classified into different blood cell lineages based on morphology and size, and enumerated in order to quantify the number colony forming cells (CFCs), representing primitive haemopoietic stem and progenitor cells, within the investigated population of cells. Although first developed in the 1960s, this assay is still widely used and even frequently applied in clinical laboratories to enumerate the functional integrity of haemopoietic stem and progenitor cells in cell preparations used for clinical transplantations. This assay was also central for the identification and characterisation of the impact of different haemopoietic growth factors on proliferation and differentiation from progenitor cells. In fact, many haemopoietic growth factors carry names that reflect this, including the cytokines granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF) and monocyte colony-stimulating factor (M-CSF).

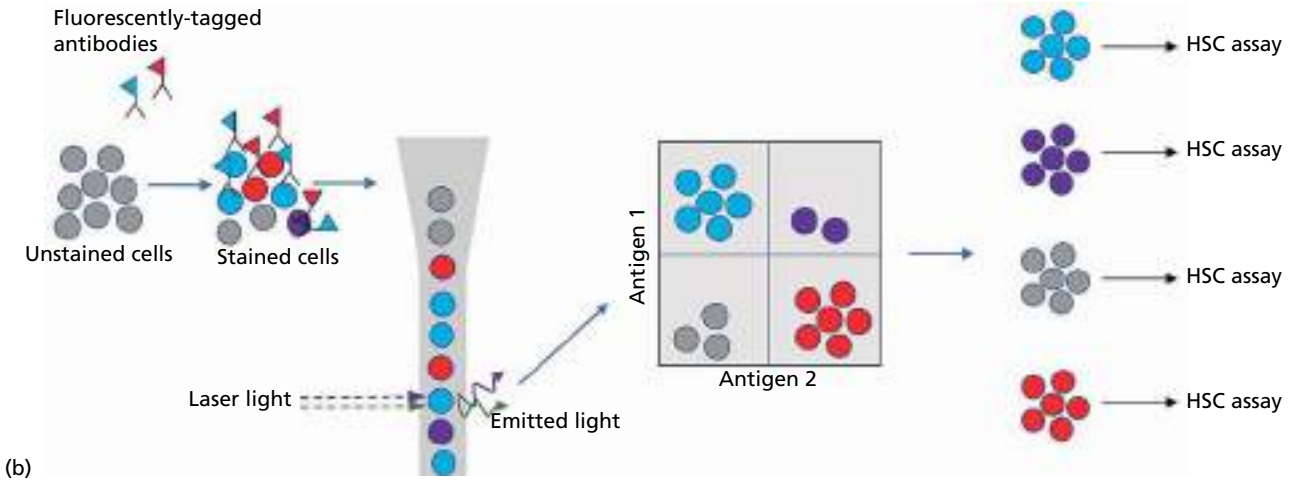
Culture of haemopoietic stem and progenitor cells in short-term liquid cultures can also be applied to evaluate their potential towards one or more lineages. These liquid culture assays come in two flavours, with or without supporting stromal cells. Although several stromal-free conditions can support the generation of myeloid and erythroid cells, the generation of lymphoid cells appears more dependent on stromal-cell support. For human stem and progenitor cells, differentiation towards B cells can be supported by OP9 stromal cells that were derived from the calvaria of newborn mice lacking M-CSF expression, whereas in order for the OP9 stromal cells to support differentiation towards the T cell lineage, transduction of the OP9 stromal cells with the Notch ligands Delta-like 1 or Delta-like 4 is required. Although culture in semi-solid medium allows quantification of single progenitors within a population, qualitative evaluation of lineage-potentials are primarily obtained from liquid cultures seeded with bulk populations. However, quantitative assessment can also be achieved by either plating cells by limiting dilution or through single-cell plating.

Long-term in vitro assays

Although useful for evaluating lineage-potentials, short-term cultures in liquid or semi-solid media are limited in their ability to evaluate self-renewal potential, and as such does not allow assessment of HSCs or long-lived progenitor cells. Instead, self-renewal potential can be assessed either by serial-replating of



(a)



(b)

Figure 1.3 Human haemopoietic stem and progenitor cell assays. (a) Overview of haemopoietic stem and progenitor cell assays where differentiation potential towards one or more blood cell lineages are explored (unipotent, oligopotent or multipotent) *in vitro* and *in vivo*. The ability to assess self-renewal potential is indicated where the scale indicates the relative longevity of the assay. Although in vitro assays and xenotransplantation are important for addressing the potential of haemopoietic cells, fate-mapping cells in human individuals is the only assay that provides information on behaviour of the cells under physiologic conditions. (b) Flow cytometry allows high-throughput analysis of protein expression on single cells within complex tissues by staining with fluorescently-tagged antibodies that bind to specific antigens expressed on the cell surface. Following exposure to specific laser light that excites the fluorescent marker, emitted light will be detected, where the level of emitted light is proportional to the number of molecules (antigens) expressed on the cell surface and bound the different fluorescently-tagged antibodies. As a result, different cells can be purified based on their cell surface expression patterns and then compared for their ability to read out in haemopoietic stem cell assays.

colonies generated in semi-solid medium, or by extended culture in liquid medium. Serial replating of colonies in semi-solid medium has in particular been applied to investigate self-renewal potential of leukemic stem cells which in contrast to HSCs, have reduced ability to differentiate and in some leukaemia can preserve their stem-like activity in the culture following several passages. Following serial replating, progenitor cells that lack self-renewal will not generate colonies that can be replated, whereas colonies derived from HSCs or leukemic stem cells can go through additional passages.

In long-term culture assays, such as the long-term culture initiating-cell assay, the ability of cells to sustain CFC activity is assessed after an initial stromal cell co-culture period of 6 weeks or longer. Following such long culture, the only cells that can

sustain ability to generate either myeloid or erythroid colonies upon transfer to semi-solid CFC culture are the cells that are endowed with extensive self-renewal potential. Cells that lack self-renewal potential, such as short-lived progenitor cells, are not able to sustain this long period of stromal cell co-culture, and therefore not able to produce colonies when transferred to the semi-solid medium. Three-dimensional scaffold cultures have also been applied to support long-term differentiation cultures.

Xenotransplantation assay

In vitro assays have been essential for the identification and characterisation of candidate human haemopoietic stem and progenitor cells, as well as providing a system where manipulations of extrinsic (cytokines, stromal cells and extracellular matrix proteins) and intrinsic (transcription factors, cytokine receptors and DNA integrity) factors can be investigated under controlled experimental settings to evaluate their impact on haemopoietic functional potential. However, mimicking the complex three-dimensional environment where HSCs reside in the human bone marrow is challenging to recapitulate with *in vitro* conditions. In particular, maintaining the quiescent nature of HSCs in adult BM has so far been unsuccessful *in vitro*. Furthermore, no single *in vitro* assays, neither after short- and long-term culture, have been demonstrated to fully address the potential to generate all blood cell lineages (multipotency). As a result, the development of an *in vivo* assay that allows assessment also of human haemopoietic stem and progenitor cells has been important.

Delineation of the haemopoietic hierarchy has been explored in mouse models, where single HSC transplantations have conclusively established the existence of truly multipotent HSCs with long-term self-renewal potential by the ability of a single HSC to reconstitute all mature lineages of the haemopoietic system, and can sustain this ability following serial transplantation. An important factor for allowing such experiments in mice is the ability to transplant donor cells that do not elicit an immunological rejection response by the recipient cells following transplantation. This has been possible through the use of mice on the same genetic background whilst still allowing the ability to distinguish between donor- and recipient-derived cells. Establishing such an *in vivo* assay for human cells, however, required further development, as it is not possible to perform similar controlled experiments in human recipients, and transplantation into wild-type mice will result in the immediate rejection of human cells due to species incompatibility (xeno-rejection). Realising the importance of an *in vivo* assay that enables investigation of human haemopoiesis, much efforts have been focused towards the development of immune-compromised mouse models that would not reject transplanted human cells.

Initial studies exploring the possibility of engrafting human haemopoietic cells in mice were performed using severe combined immune-deficient (*Scid*) mice that lack B and T cells. These studies demonstrated that human cells indeed could engraft in immunocompromised mice but it was not possible to demonstrate whether engraftment originated from multipotent human HSCs, as only lymphoid cells were observed in these early studies. Only by combining transplantation of human cells with infusion of human haemopoietic growth factors, including interleukin 3 (IL-3), GM-CSF and stem cell factor (SCF) was the long-term generation of lymphoid and myeloid cells observed, supporting that also multipotent and long-lived human stem or progenitor cells can engraft mice. However, as Scid mice were not fully immunocompromised, with spontaneous emergence of both mouse B and T cells, the immunocompromised mouse models have gone through series of genetic modifications to further enhance their ability to support long-term *in vivo* studies of human cells.

By crossing Scid mice to nonobese diabetic (NOD) mice to generate NOD/Scid mice, the rejection mediated by the mouse innate immune system was inhibited as the protein Sirpa expressed on NOD/Scid myeloid cells could bind to CD47 expressed on human cells, thereby preventing phagocytosis of human cells. Additionally, the NOD/Scid mice have been further modified to ablate expression of the common γ chain receptor, critical for transmitting signals from haemopoietic growth factors required for lymphoid differentiation. In addition to being more immunocompromised than the NOD/Scid mice, as NOD/Scid/common γ chain knockout (NSG) mice lack B, T and NK cells, the NSG mouse model also allows for more long-term studies as the risk for spontaneous lymphoma development is dramatically reduced in comparison to what seen in ageing NOD/Scid mice. Both NOD/Scid and NSG mice support long-term multipotent differentiation from transplanted human HSCs, including development of mature myeloid, lymphoid and erythroid cells. However, as several mouse haemopoietic growth factors are not cross-reactive to human cells, several critical components for supporting maintenance, propagation and differentiation of human haemopoietic cells are lacking. As a result, even newer generations of mice have been developed where factors supporting human haemopoietic growth and differentiation are expressed in the mouse bone marrow environment, including haemopoietic growth factors, as well as human leukocyte antigens (HLA) critical for allowing generation of mature T lymphocytes.

Fate-mapping

The use of immunocompromised mice has enabled *in vivo* studies of human HSC and progenitor cells, even down to the single cell level, and as such represent an essential tool for the characterisation of human haemopoiesis. However, this still is an assay where human cells are transferred into a stressed environment which lack several factors critical for supporting human haemopoiesis due to species incompatibility. As a

result, xenotransplantation does not necessarily reflect the behaviour of human cells under normal physiological conditions in the human bone marrow. Studies on mouse haemopoiesis using elegant genetically-modified mouse models have highlighted differences in the contribution of mouse haemopoietic stem and progenitor cells to active blood production under normal unperturbed conditions (steady-state) as compared to following stress, such as after transplantation. These studies, where the fate of different haemopoietic stem and progenitor cells are tracked over time (fate-mapping), have until recently been challenging to apply to studies of human haemopoiesis as the they depend on genetic modification of the mouse genome to generate suitable mouse strains for this purpose. However, with the advances in next-generation DNA sequencing, fate-mapping approaches can now be applied to study human haemopoiesis. Although X chromosome inactivation provided some insights, the advancement of DNA sequencing has allowed the ability to track the fate of single HSCs and long-lived progenitor cells within an individual, as will be explained in a separate section later.

Identification of human haemopoietic stem cells

As HSCs are rare in adult bone marrow, where as few as only one in a million bone marrow cells in human bone marrow represent a transplantable HSC, further enrichment is required in order to separate these cells from other cells in the bone marrow. In addition to the assays for evaluation of the functional behaviour of human haemopoietic cells, the development and application of fluorescently tagged antigen-specific antibodies combined with fluorescent activated cell sorting (FACS), has allowed the prospective identification and purification of haemopoietic stem and progenitor cells with different functional characteristics (Figure 1.3b). As haemopoietic cells in bone marrow and peripheral blood already are in single-cell suspension and easily accessible for collection from healthy and diseased individuals, these are particularly amenable to FACS. By staining haemopoietic cells with fluorescently tagged monoclonal antibodies recognising and specifically binding cell surface antigens, the protein expression on thousands of single cells can be analysed per second and viable cells with different expression patterns can be extracted for investigation in different haemopoietic assays. In initial studies, only a few cell surface markers could be investigated simultaneously, but with development of new fluorescent colours that can be tagged to antibodies and separated from one another based on the wavelength of emitted light following excitation of focused laser lights, current technology allows for the simultaneous analysis of more than 20 cell surface markers on single cells.

By combining FACS with the haemopoietic assays described above, it has been possible to enrich cells with HSC properties based on their cell surface expression pattern. In addition to enriching human HSCs by exclusion of known markers expressed on mature blood cells (so-called lineage markers), expression of the cell surface glycoprotein CD34, expressed by approximately 5% of the cells in adult human bone marrow, allows for purification of human HSCs. Although studies have reported that some human HSC activity can be found also outside of the $CD34^+$ cells, it is proposed that at least 99% of human HSCs are confined within the $CD34^+$ compartment. However, as the majority of the $CD34^+$ cells represent non-HSCs, including multipotent and oligopotent progenitor cells, additional markers have been identified to further separate HSCs from $CD34^+$ non-HSCs. The culmination of this work has led to the current phenotype allowing for the highest enrichment of human HSCs from both cord blood and adult bone marrow to encompass cells that lack expression of lineage markers ($Lineage^-$), are positive for CD34 and CD90, and negative for CD38 and CD45RA. By further sub-fractionating $Lineage^-CD34^+CD38^-CD90^+CD45RA^-$ cord blood cells-based expression of CD49f and ability to exclude the triarylmethane dye Rhodamine, a further enrichment can be achieved, where 14–28% of $Lineage^-CD34^+CD38^-CD90^+CD45RA^-CD49f^+Rhodamine^-$ cord blood cells were able to long-term reconstitute myeloid and lymphoid lineages following single cell transplantation into NSG mice. To date, this represent the most stringent and highest enrichment of human HSCs that have been demonstrated experimentally through single cell transplantation, where the inclusion of additional candidate markers, such as EPCR, could allow even further enrichment of bona fide human HSCs.

The application of cell surface molecules to enrich for human HSCs will be important for further functional characterisation of human HSCs. Although previously viewed as a relatively homogenous population, the mouse HSC compartment has recently been shown to include multiple HSC subsets, all endowed with long-term self-renewal potential but with different preferences in their contribution towards mature blood cell lineages. Single mouse HSC transplantation experiments have revealed that all mouse HSC subsets actively contribute to the platelet lineage, but with the exception of HSCs contributing to all mature blood cell lineages, these studies have demonstrated that the adult mouse bone marrow contains a high frequency of HSCs which lack contribution towards one or more blood cell lineages. HSCs exclusively contributing to mature platelets were the only uni-lineage HSC detected in these studies. Platelets generated from megakaryocytes are critical for preserving normal body function and life through their ability to prevent life-threatening bleeding. Mature megakaryocytes undergo multiple rounds of endomitosis, increasing ploidy and cytoplasm to ultimately allow platelet formation (see Chapter 40 for

more details on platelet formation and function). Although platelets are the second most abundant cell in blood (after red blood cells), they have the shortest lifespan and critically dependent on efficient production from HSCs. Interestingly, both in human and mouse haemopoiesis alternative differentiation pathways for the generation of mature platelets from haemopoietic stem and progenitor cells have been proposed, including so-called emergency pathways which bypass intermediate multipotent progenitor cell stages and allow the generation of megakaryocyte progenitor cells directly from HSCs. The identification of human HSC subsets generating platelets through alternative and potentially faster differentiation pathways has clinical relevance as these represent candidate pathways that can be exploited therapeutically to expedite platelet production in patients with life-threatening platelet deficiencies.

The ability to enrich human HSCs has not only been important for allowing cellular, molecular and functional characterisation of human HSCs, but has also been applied in clinical practice. Allogeneic bone marrow transplantation, where the donor bone marrow originates from a person that has a similar but not fully identical HLA match to the recipient, carries the risk of promoting a graft-versus-host-disease (GvHD) mediated by the donor T cells included in the transplant which mediate an allogeneic immune reaction against the recipient's own cells. In the worst case, such a reaction can have deadly outcome. In an effort to minimise the severity of the GvHD reaction, identifying a way to reduce the number of T cells included in the transplant without impacting the number of HSCs required for the reconstitution of recipient haemopoiesis was explored. Towards this, CD34 enrichment is sometimes used for this purpose, allowing the simultaneous enrichment of HSCs and depletion of T cells from whole bone marrow, as well as peripheral blood following donor-conditioning with a HSC mobilising agent that recruits $CD34^+$ cells from the bone marrow into peripheral blood.

Development of haemopoietic stem cells

Although the bone marrow is the primary site of HSCs and blood cell production in the adult, the emergence, expansion and differentiation of HSCs takes place at different sites and time points during embryonic development. Studies in mice and human embryos have shown a conserved appearance of the first haemopoietic cells, which are first detected in the human extra-embryonic yolk sac already 16–18.5 days post-conception. However, these cells lack bona fide HSC activity as the direct isolation of cells capable of reconstituting all blood lineages so far has not been possible from these first haemopoietic cells found in the developing yolk sac and are referred to as primitive haemopoiesis. Instead, the first HSCs and definitive haemopoiesis originate from within the human embryo around 19–27 days post-conception, where emerging haemopoietic cells can be found as budding off from endothelial cells lining the dorsal aorta in the aorta-gonad mesonephros region. Upon establishment of circulation, these cells migrate to and seed the fetal liver, which represent the major site of HSCs and haemopoietic differentiation during the embryonic period. In contrast to the highly quiescent HSCs in adult bone marrow, HSCs are during embryonic development highly proliferative and to a large degree undergo symmetric self-renewal divisions resulting in the rapid expansion of the number of HSCs in the embryo. This is critical, as the pool of HSCs required to sustain haemopoiesis through adult life are formed during this period. Shortly before birth, HSCs migrate from the fetal liver and other embryonic haemopoietic sites to seed the bone marrow which now takes over as the primary haemopoietic organ. In the mouse, bone marrow HSCs still remain in a proliferative phase for a period of around 3–4 weeks after birth, whereupon they enter the quiescent phase of the cell cycle and remain largely dormant unless stimulated to exit quiescence following exposure to stress.

In light of the challenges with recovery of sufficient HSCs for clinical application, including bone marrow transplantation, the mechanisms regulating the distinct proliferative states and symmetrical self-renewal divisions during embryonic and adult haemopoiesis has been a major focus. Uncovering the conditions and regulatory mechanisms that allow for rapid HSC expansion without loss of multipotency and self-renewal potential represents a key to unlocking the ability to expand HSCs for clinical use. Recent techniques have been developed which allow the *ex vivo* expansion of human and mouse HSC and now awaits clinical validation. Several transcription factors have been implicated towards this and this remains a major interest for translational biologists. Although the site of HSC and active haemopoiesis does not change following birth, ageing is associated with altered HSC performance and output. Both in mice and humans, the number of phenotypic HSCs expands with age. However, they appear to loose both proliferative potential and capacity for multi-lineage differentiation. In particular lymphoid potential is reduced with ageing, resulting in skewed myeloid differentiation from aged HSCs. Similarly, the frequency of single mouse HSCs capable of reconstituting mouse haemopoiesis declines with age.

Regulation of haemopoietic stem cells

The preservation of the normal HSC pool in the adult bone marrow, including maintaining their predominant quiescent state during steady-state haemopoiesis and the activation of

HSCs in response to stress, is tightly controlled by a complex interplay of intrinsic (internal to HSCs) and extrinsic (environmental) factors. This tightly regulated process is critical for protecting the HSCs from exhaustion or expansion, and dysregulation of factors impacting on HSC self-renewal and multi-lineage differentiation potential are frequently seen in haematologic diseases and malignancies.

Several intrinsic factors have been demonstrated to be critical for maintaining HSC self-renewal potential and ability to generate all blood cell lineages (multipotency). Particularly through work in genetically modified model organisms, the role of several transcription factors for ensuring HSC self-renewal capacity (e.g. Pbx1 and Evi1) and quiescence (Egr1, Gfi1 and FoxO) has been identified, where deficiency of these factors leads to HSC exhaustion as revealed through loss of ability to undergo serial transplantation in mice. Furthermore, epigenetic regulators involved in reshaping accessibility of the genome and the transcriptional activity, including the methyl-cytosine dioxygenase TET2, DNA methyltransferase 3A (DNTM3A) and Polycomb repressive complex members such as EZH2, are critical for regulating HSC activity. The importance of transcription factors and epigenetic regulators, as well as their impact on haemopoiesis, is demonstrated by the recurrent nature of mutations targeted to these genes in haematologic malignancies, leading to clonal outgrowth of mutated cells and reduced differentiation potential.

In addition to the tight transcriptional regulation of HSCs, it is also critical to protect the genomic integrity in HSCs. Cell division requires replication of DNA and although DNA damage repair mechanisms are critical for minimising the number of mutations acquired by each cell division, it is estimated that each human HSC accumulate on average 17 mutations per year. In addition, cell divisions result in shortening of telomere length. Both excessive DNA damage and telomere shortening are associated with HSC exhaustion. Maintaining HSC quiescence is, therefore, a key mechanism for preserving the HSC genome and maintenance of normal HSC activity. In addition to genetic factors controlling HSC metabolic activity where quiescent HSC undergo glycolysis, rather than the mitochondrial oxidative phosphorylation-driven metabolism utilised by actively dividing cells, the DNA damage repair machinery safeguards the genomic integrity in HSCs. In conditions where components of the DNA damage repair pathway are mutated, such as in Fanconi anaemia, the ensuing genomic instability results in a progressive HSC dysfunction leading to bone marrow failure, where bone marrow transplantation remains the only curative option.

As described above, the use of *in vitro* cell culture assays has facilitated the identification of haemopoietic growth factors supporting differentiation, as well as maintenance of HSCs. Although several of the haemopoietic growth factors might be produced from haemopoietic and non-haemopoietic cells residing in the bone marrow, there are also several growth factors critical for bone marrow haemopoiesis that are secreted from non-haemopoietic cells outside of the bone marrow. Thrombopoietin, a cytokine required to maintain bone marrow HSC function and preserve HSC quiescence is secreted by hepatocytes in the liver. This cytokine therefore regulates HSC by binding to and activating the thrombopoietin receptor (MPL) expressed on the HSC cell surface primarily through systemic, rather than local, delivery. In addition to being a critical cytokine for supporting HSC self-renewal *in vitro*, the critical role for preserving HSCs is demonstrated in human individuals with congenital amegakaryocytic thrombocytopenia, where inherited mutations in the thrombopoietin receptor results in HSC deficiency and multilineage haemopoietic defects which only can be rescued by bone marrow transplantation.

Another critical cytokine for regulating HSC activity is stem cell factor (SCF, or Kit-ligand). In mice, mutations in both SCF and the receptor for SCF, KIT, results in HSC loss, and the importance of SCF in supporting HSC self-renewal has also been supported by *in vitro* cultures. Interestingly, SCF can be produced as both a secreted and membrane-bound form, where in fact the membrane-bound form is believed to be particularly important for regulating HSC activity and in contrast to thrombopoietin acting primarily through local effects in the bone marrow microenvironment.

In addition to the haemopoietic growth factors, several components surrounding the HSCs in the bone marrow are important for maintaining the HSCs in their quiescent state, including interaction with extracellular matrix components and direct cell-to-cell interactions with niche components such as endothelial cells and mesenchymal stromal cells. Genetically modified model systems combined with advanced imaging technology, allowing high resolution imaging and localisation of distinct cellular and molecular components in the bone marrow, have revealed that HSCs are not randomly distributed throughout the bone marrow microenvironment, but rather appear physically embedded within distinct anatomic locations. This has resulted in the hypothesis that HSCs reside and depend on a distinct bone marrow niche, composed of a defined set of cell types responsible for maintaining the HSC pool through local interactions. These studies suggest a complex interplay among different cellular and secreted factors. Furthermore, it remains unclear to what degree HSC depend on a specific anatomically defined niche cell type or factor as HSC can be detected outside of the bone marrow microenvironment. During embryonic development, several sites can support HSC propagation, and functional HSCs can be isolated from peripheral blood. The latter is exploited in clinical practice, where stimulation with G-CSF or compounds that disrupt interaction between stromal-derived factor (SDF) and the chemokine receptor CXCR4, result in mobilisation of transplantable HSCs from bone marrow to the peripheral blood.

Fate-mapping human haemopoiesis

The delineation of the human haemopoietic hierarchy has so far depended on experimental assays where the potential of human cells to sustain self-renewal and multi-lineage differentiation has been explored following *in vitro* culture or transplantation into immunocompromised mice. Although these assays can provide important insight towards the functional potential of the investigated human cells under these experimental conditions, it is also important to remember that these represent artificial assays and conditions that do not necessarily reflect the natural habitat of human cells. Furthermore, the behaviour of the human cells under these conditions, might not reflect their actual behaviour or fate under normal physiological conditions in the human body. Such studies require fate-mapping approaches whereby the contribution (multipotency) and longevity (self-renewal) can be monitored under normal unperturbed conditions, as well as under stress. Genetically modified model organisms have provided important insights to both establishment of the haemopoietic system during embryonic development, as well as the contribution of distinct stem and progenitor cells towards steady-state haemopoiesis. Until recently the possibility to apply such approaches to human haemopoiesis was limited. However, with advances in next-generation DNA sequencing, it is now also possible to apply this approach and track the fate of single haemopoietic stem and progenitor cells in human subjects through genetic fate-mapping (Figure 1.4).

Genetic fate-mapping relies on the inheritance of the parental genome to each generated daughter cell, and by utilising genetic marks unique to each single parental cell, the contribution of one original cell can be investigated over time (Figure 1.4a). If a genetic mark is targeted to a parental cell with self-renewal and multi-lineage potential, the genetic mark will be inherited to the downstream daughter cells, as well as any progeny generated from these, and be sustained over long period of time. However, if a genetic mark is targeted to a cell that lacks self-renewal ability, this genetic mark will be lost over time when the parental cell reaches its defined lifespan. Furthermore, if targeted to a downstream progenitor cell, the genetic mark will not be detected further upstream in the hierarchy as differentiation is unidirectional.

In model organisms, trackable genetic marks have been introduced to the genome through elegant genetic engineering approaches, which allows introduction of genetic marks to specific cellular compartments and different time points during embryonic or adult development. In human haemopoiesis, a similar strategy can be achieved in clinical gene therapy trials, whereby the integration site of the lentiviral vector carrying the modified gene into the genome of the haemopoietic stem and progenitor cells transplanted into the patient provides a trackable genetic marker (Figure 1.4b). As the lentiviral integration site is unique for each donor HSC and progenitor cell transplanted into the patient, the single cell fate can be tracked following transplantation by DNA sequencing strategies that allow quantification of each unique integration site in different cellular compartments at different time points following transplantation. A donor cell that has self-renewal, multipotency and active contribution to haemopoiesis will result in high detection levels of specific integration site in all nucleated blood cell lineages and over extended period of time. In contrast, a unique integration site targeted to a progenitor could perhaps be detected at an early time point following transplantation, but would be lost at later time points. Such studies have revealed that short-lived progenitors can contribute to multi-lineage reconstitution at early stages following transplantation, but upon long-term follow-up these are lost, and contribution from HSC takes over as the major source of post-transplantation haemopoiesis.

Although tracing genetically distinct haemopoietic clones in gene therapy trials allows assessment of haemopoietic stem and progenitor contribution to distinct blood cell lineages, this strategy depend on *ex vivo* genetic modification of haemopoietic stem and progenitor cells. Furthermore, the conditioning associated with transplantation can alter the bone marrow environment and activate otherwise dormant HSCs, and as such, might not reflect their contribution or behaviour during steady-state haemopoiesis. In order to accomplish genetic fate-mapping of normal human haemopoiesis, one therefore needs to obtain reliable genetic markers that can be tracked without any *ex vivo* manipulation or introduction of cellular stress. Due to imperfect DNA repair mechanisms, each cell will acquire new DNA mutations with each DNA replication and it is estimated that human HSCs accumulate on average 17 mutations per year. The vast majority of these mutations are targeted to non-coding regions of the genome and has no functional impact on cell biology. As these mutations will be inherited each time the cell divide, the mutations will be transferred to all generated daughter cells. These somatically acquired mutations therefore represents unique genetic marks or barcodes that can be exploited for fate-mapping approaches (Figure 1.4c), where the number of mutations detected for each single cell also provide insight to the longevity of the unique clone. By first performing genetic profiling of single cells by DNA sequencing, the genetic profile (barcode) of a single cell can be established. The contribution of cells with unique genetic barcodes can then be quantified within different cell compartments in the haemopoietic hierarchy. Advanced computational analysis of the contribution of distinct genetic barcodes has also allowed predictions over the expansion and size of the HSC pool during early stages of embryonic development up to old age. Current estimates suggest that adult human normal bone marrow contain 50,000–200,000 HSCs that actively contributes to haemopoiesis. Further advances in DNA sequencing and computational analysis will likely continue to provide new and important insight to the establishment and maintenance of the human haemopoietic system, during steady-state, during stress and in haematologic disease.

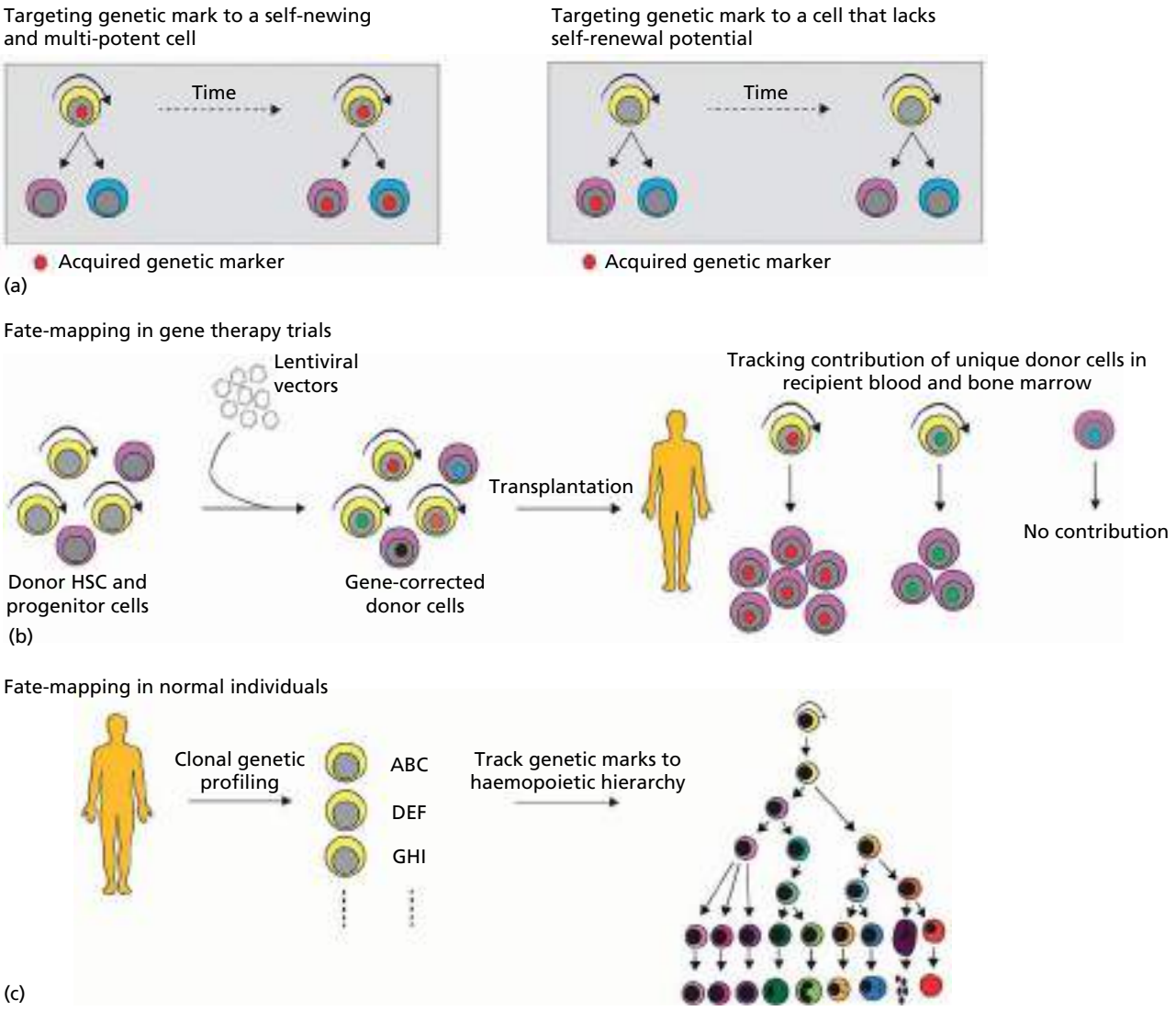


Figure 1.4 Genetic fate-mapping of human haemopoiesis. (a) Overall principle of genetic fate-mapping approaches where a permanent acquired genetic mark is tracked within different haemopoietic compartments over time. If the genetic mark is introduced to a self-renewing and multipotent haemopoietic stem cell, the genetic mark will be inherited to the downstream progeny and be detected over a long period of time. However, if a genetic mark is acquired in a cell that lacks self-renewal, such as a downstream progenitor cell, the genetic mark will not be sustained over time. (b) As a result of the random integration of the lentiviral vectors in the human genome, the integration site of can be used to track the fate of individual cells originating from the gene corrected pool of cells transplanted into the patient. The contribution to different stem/progenitor and mature lineages can be tracked over time, allowing identification of clones which actively contribute to haemopoiesis following transplantation. (c) Errors in DNA replication results in accumulation of DNA mutations which are unique to individual cells, providing a genetic barcode that can be tracked through DNA sequencing. Similar to viral integration sites, the fate of the different mutations/barcodes can be investigated in different cellular compartments and over time.

Clinical relevance of haemopoietic stem cells

Dysregulation of HSCs is linked to development of haematologic disease and leukaemia, where HSC exhaustion is associated with cytopenia and HSC expansion is associated with risk for leukemic transformation. In light of the regenerative potential for HSCs, their clinical relevance for potentially correcting conditions with dysregulated haemopoiesis has been explored through bone marrow and HSC transplantations. HSC transplantation represent to-date the only curative treatment for several haematologic malignancies and diseases. However,

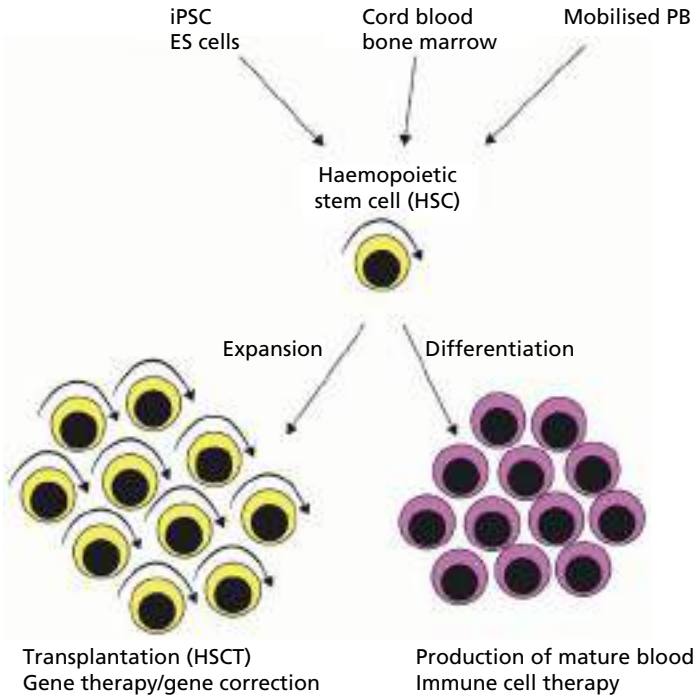


Figure 1.5 Clinical use of haemopoietic stem cells. Haemopoietic stem cells are already widely used in the clinic to replace or correct diseased haemopoiesis following transplantation and/or gene therapy. Sources of haemopoietic stem cells are limited, currently originating from matched donor cord blood, bone marrow or mobilised peripheral blood. The *ex vivo* expansion of HSCs from such sources or from induced pluripotent stem cells or embryonic stem cells could in the future expand the availability of this treatment to more patients. Furthermore, as differentiated progeny such as red blood cells, platelets and immune cells are used clinically, the *ex vivo* production of these could be expanded for future use.

access to sufficient and suitably matched donor HSCs still remains a challenge for the extension of this therapy to all patients. Both autologous and allogeneic bone marrow transplantation is used for a wide range of haematologic conditions, where autologous transplantations are favoured in gene therapy trials, whereas allogeneic transplantations are favoured for treatment of leukaemia due to their associated graft-versus-leukaemia effect. In addition, cord blood and mobilised peripheral blood represent additional sources of HSCs for clinical use in transplantation (Figure 1.5).

In allogeneic transplantations, careful matching of donor human leukocyte antigen (HLA) profile to that of the patient's HLA pattern is critical to avoid overactivation of donor-derived immune cells towards the patient's own cells, resulting in GvHD. As a result, both the expansion of HSCs *ex vivo*, as well as the generation of HSCs from alternative sources that could represent more universal source of donor HSC, such as generation from embryonic stem cells or induced pluripotent stem cells, compared to HSCs isolated from healthy individuals, still represent attractive options for expanding the clinical use for HSC therapies. Furthermore, considering the multi-lineage differentiation potential, HSCs also represent a source that can generate mature blood cell products for clinical use, including platelets and red blood cells following acute bleeding, and immune cells for immune therapies. Much research is therefore focused at *ex vivo* conditions that allow for the expansion and/or differentiation of HSCs for clinical use.

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CHAPTER 2

Phagocytes

2

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With thanks to John Mascarenhas, Marina Kremyanskaya and Ronald Hoffman from the Icahn School of Medicine at Mount Sinai, New York, USA for the previous version of the Chapter.

Introduction

Phagocytes owe their name to their ability to ingest and destroy material such as pathogens and dead cells. Professional phagocytes include granulocytes and mononuclear cells. The granulocyte family includes acute-acting cells such as Neutrophils, Eosinophils, Basophils and Mast cells, exceptionally equipped to deal immediately with intracellular and extracellular pathogens, their granules loaded with preformed antimicrobial proteins. The mononuclear myeloid leucocyte family referred to as the Mononuclear phagocyte system (MPS), includes Monocytes, Dendritic cells (DCs), tissue Macrophages and Multinucleated Giant Cells in tissues and bone.

Granulocytes and MPS cells are versatile cells of the immune system and their heterogeneous functions span homeostasis, acute and chronic inflammation, host defence against infection and repair after injury. In the steady state, they clear senescent and apoptotic cells as part of their trophic functions in tissue growth and remodelling. Phagocytes play an essential role in innate and adaptive immunity; they recognise, kill and clear invading microorganisms, and nurse as well as destroy tumour cells. Phagocytes produce a variety of secretory products, including enzymes, cytokines, chemokines and metabolites, that regulate tissue functions, mediating cellular and humoral responses such as hypersensitivity and allergy. In this chapter, we consider the steady state production and function of phagocytes, biomarkers to study their properties and describe molecular pathways that underlie their properties. Finally, we address defects in molecules of the phagocyte, from proliferation in bone marrow, to migration, phagocytosis and microbial killing.

The phagocyte lineage at a glance

Phagocytes originate in bone marrow, from quiescent long-term haematopoietic stem cells (LT-HSC), through short-term HSC (ST-HSC) and haematopoietic multipotent progenitor (MPP) cells and more committed progenitor and precursor cells (Figure 2.1; see also Chapter 1). Subsets of MPPs give rise to the lineage-committed common myeloid progenitors (CMP; surface markers: Lin-CD34+CD38+IL-3Rα+CD45RA-) that further differentiate into the granulocyte macrophage progenitors (GMP; Lin-CD34+CD38+IL-3Rα+CD45RA+). CMP are defined as CFU-GEMM (colony-forming-unit–granulocyte, erythrocyte, monocyte, megakaryocyte), whilst the more committed GMP is termed CFU-GM (granulocyte, monocyte) (Figure 2.1). Downstream of the myeloid progenitors, myeloblasts give rise to all circulating phagocytes.

Lineage cytokine receptors drive the commitment of precursors. Granulocyte development and proliferation are largely driven by Colony Stimulating Factor 3 (CSF3), better known as Granulocyte colony-stimulating factor G-CSF. CSF3 binds to CSF3R (CD114, G-CSFR), a homodimer receptor of the

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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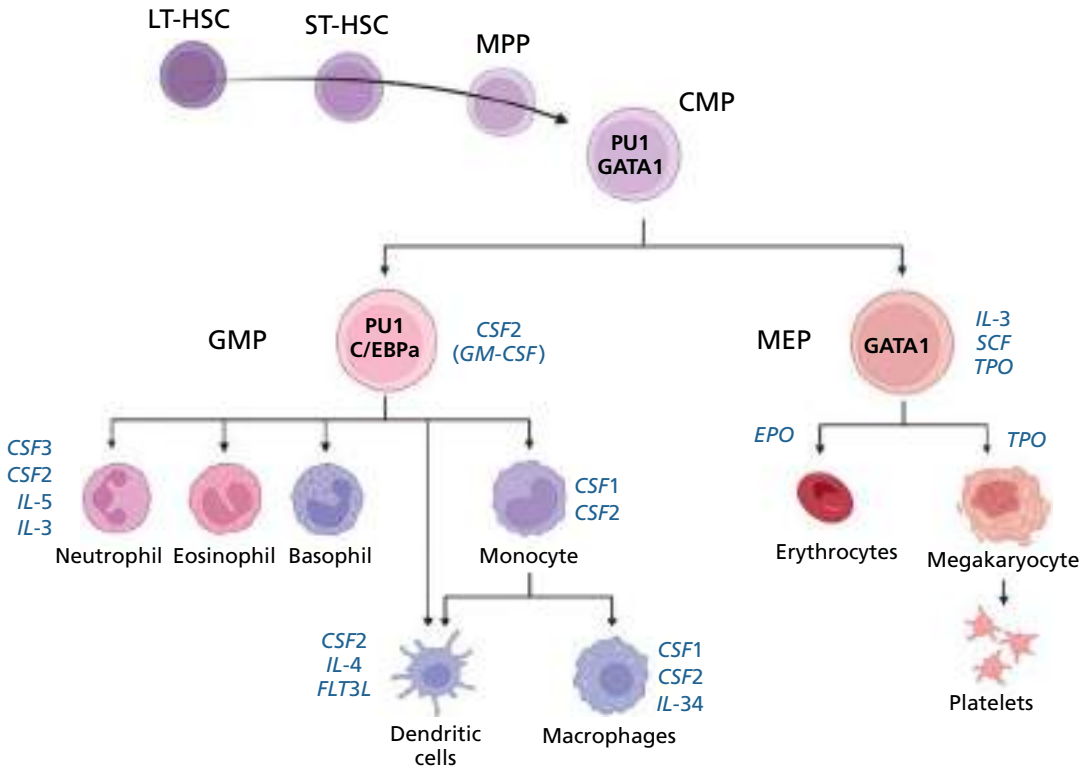

Figure 2.1 Haematopoiesis is controlled by lineage-determining cytokines and specific transcription factors. Stem cells progress down the line of mature haematopoietic cells with the aid of cytokines present in the bone marrow milieu. How specific stem cells acquire the expression and activate specific lineage receptors is until now considered a stochastic process. In this figure, the main stages are highlighted, the transcription factors appear in bold and the cytokines that drive the commitment in blue. PU1 Is the most important transcription factor for the phagocyte family. M-CSF, GM-CSF and G-CSF control the differentiation to monocyte, neutrophil or DCs.

cytokine family, that signals through the JAK/STAT pathway. CSF3R is highly expressed in mature neutrophils. Monocytes and macrophages are controlled by CSF1 (macrophage colony-stimulating factor M-CSF). CSF2 (Granulocyte-macrophage Colony stimulating factor GM-CSF) is important for both monocytes and granulocytes. CSF2 binds its receptor CSF2R (CD116, or GM-CSFR), a Type I interleukin receptor. CSF2 is a potent inflammatory cytokine contributing to damage in auto-inflammatory conditions. CSF1 promotes monocyte survival and stimulates macrophage maturation by binding to its receptor CSF1R CD115, or M-CSFR, a homodimeric Type III receptor tyrosine kinase of the PDGF family. CSF1, together with Receptor activator of nuclear factor kappa-B ligand (RANKL), are associated with osteoclast development in bones. IL-34 is another ligand for CSF1R in the skin and brain, where it is involved in the development of the resident MPS cells, the Langerhans cells and microglia, respectively. Other important receptors for phagocytes are FLT3 (CD135) and stem cell factor receptor (SCFR, CD117 or cKIT). SCFR is involved in early haematopoiesis, at the stem cell stage, and the development of mast cells. FLT3 is involved in early cell differentiation and in DC development. IL-5 is the main lineage cytokine for eosinophils. IL-3, a lineage cytokine involved in the early stages of haematopoiesis, drives development of several haematopoietic cells.

Granulocytes are the most abundant phagocytes in blood. They are terminally differentiated cells, and with the exception of mast cells, they tend to have a short life span. Neutrophils are the most abundant in human blood, making up 50–70% of all leucocytes. Due to their short half-life, coupled with their requirement, 1 billion neutrophils must be produced per day per kilogram of body weight to maintain steady-state conditions in humans. Eosinophils make up 1–4% of all leucocytes in the steady state. Eosinophils also derive from bone marrow stem-cell-derived myeloid progenitors in response to IL-3, GM-CSF and IL-5. IL-5 is the most eosinophil-specific factor and plays an important role in regulating terminal differentiation and post-mitotic activation of the cells. Basophils make up only 0.5–1% of all circulating leucocytes. They develop under the influence of IL-3 in the bone marrow and circulate in the blood. They specialise in fighting helminth infections. Mast cells, which derive

from CD34+, c-Kit-positive progenitors, leave the marrow before terminal maturation and home to vascularised peripheral tissues, where they mature. Mast cell growth and development require the presence of SCF.

The MPS is a more complex system and the first phagocytes to appear are macrophages, arising from the endothelium of the dorsal aorta, gonad, mesonephros (AGM) region, appear in the yolk sac, and in later weeks, in the fetal liver. Embryonic macrophages give rise to resident tissue macrophage populations which persist in many organs after birth. Embryonic yolk sac-derived macrophages/fetal liver monocytes migrate to the brain (microglia), heart and skin epidermis (Langerhans cells). After birth, there is an initial expansion of monocytes which become the most abundant blood MPS cell, representing 2–8% of the total number of leucocytes. Monocytes are differentiated cells but have the ability to enter tissues and mature into macrophages.

DCs appear in subsets according to the expression of membrane markers. The main types are myeloid/conventional DC1 and DC2 and plasmacytoid DC (pDC). DCs are less abundant in the circulation and usually represent 0.1–0.35% of mononuclear cells. Myeloid and plasmacytoid DCs are usually referred to as peripheral blood DC, because the largest DC pool resides in tissues. Precursor DC are released from the bone marrow and enter tissues in which they differentiate into functional antigen-presenting DCs. DC-specific cytokines include FLT3, IL-3 and IL-4. Finally, also in the MPS, multinucleated giant cells arise by fusion of monocyte/macrophages to form osteoclasts in bone, giant cells in granulomata and in response to foreign bodies as well as adipoclasts in adipose tissue.

Phagocyte identification and quantification

Phagocytes are routinely enumerated in the clinic for diagnosis, prognosis and treatment of a variety of pathologies. With microscopy (Figure 2.2), neutrophils appear as small granular cells with a multi-lobulated nucleus, eosinophils with red granules and a bilobulated nucleus, and basophils with purple granules. Monocytes instead appear as slightly larger cells with no evident granules, a kidney shape and less condensed nucleus. By electron microscopy the features can be discerned in more detail. Although many pathologies still need confirmation by microscopy, the Coulter counter and other modern forms of haematology analysers enable faster and more accurate measurement of complete blood counts. These instruments provide cell numbers, cell size and cell width. Cell size can be influenced by infection, apoptosis, phagocytosis and steroid treatment. An increase in monocyte width for example can be an early indicator in sepsis. Many parameters beyond cell numbers can be included in the Coulter output including cell volume, nuclear morphology, cell width and cytoplasmic light scattering.

For research and clinical applications, flow cytometry can be used to distinguish phagocytes (FACS-Flow assisted cell sorting), Figure 2.3. Neutrophils are most granular and large, followed by monocytes that adopt an intermediate position. HLA-DR staining further separates monocytes and DCs versus neutrophils. In a single step, neutrophils can be recognised by the expression of CD15 Lewis x antigen, a fucosylated antigen, posttranslationally modified by fucosyltransferases. Whole blood staining for Siglec-eight labels a glycan moiety, selective for eosinophils; it is an inhibitory receptor that induces apoptosis in eosinophils, important to control excessive tissue damage. CD123 (ILR3A) is the main cytokine receptor driving commitment of basophils and plasmacytoid DCs. An alternative method to distinguish eosinophils and basophils from neutrophils and monocytes by FACS is use of the IL-5R. Cells of the MPS in blood can be distinguished by labelling CSF1R. Further staining with CD14, an LPS receptor, and CD16, an Fc receptor, separates monocytes into their main subsets. CD1c, marker for the main DC subsets, is a transmembrane glycoprotein presenting lipids at the cell surface for recognition. FACS and conventional microscopy have improved in recent years; Mass Cytometry applications such as CyTOF (Cytometry by time of flight) and imaging, as well as in situ RNA sequencing and single-cell RNAseq, make it possible to investigate many markers and cells at the same time.

General phagocyte molecules and functions

Phagocytosis

High phagocytic ability distinguishes phagocytes from other cells. In the steady state, efferocytosis or elimination of apoptotic cells is one of the main functions of phagocytes. The membrane of apoptotic cells binds increased amounts of thrombospondin, a macrophage secretory product recognised by the scavenger receptor CD36, thrombospondin receptor, and integrin αvβ3, vitronectin receptor. CD36, in addition, recognises the exposed phosphatidylserine. Other phagocytic lectin receptors bind carbohydrate determinants exposed on the surface of apoptotic cells. Conserved apoptotic cell-associated molecules are recognised by innate immune receptors such as CD14, CD91, C1q, C3bi, collectins and pentraxins.

During infection, phagocytosis is mediated by the recognition of ligands of pathogens and is enhanced by opsonins, soluble receptors that serve as a bridge between the particle and specialised cell receptors, listed in Table 2.1. Opsonins include immunoglobulin molecules such as IgA, IgE and IgG and the complement cascade elements C3b, C3bi, C3d and C4b, recognised by the CR1 and CR3 complement receptors. Phagocytosis can also be mediated directly by non-opsonic phagocytic receptors. These receptors recognise microbial

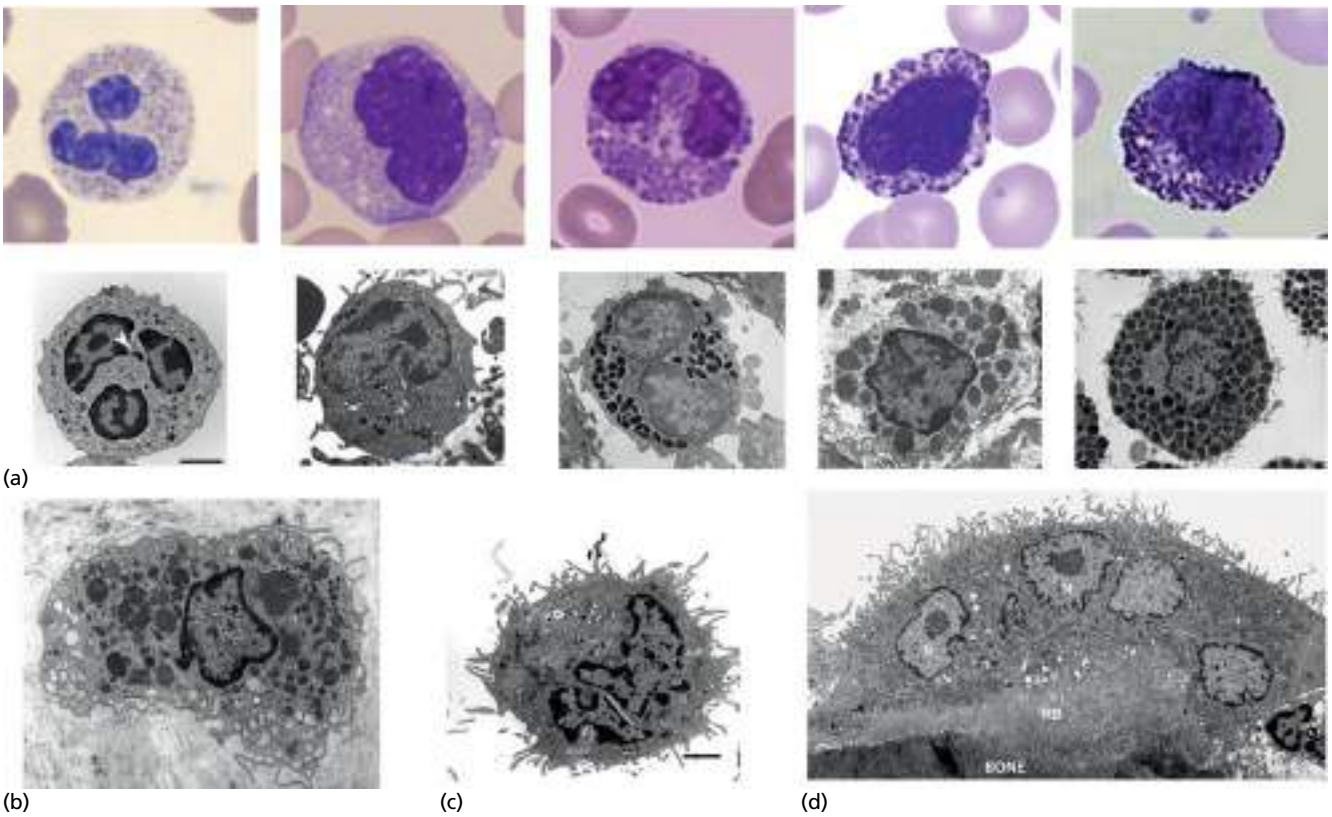

Figure 2.2 Morphology of the main phagocytes. (a) PMN or neutrophils appear with a multi-lobulated nucleus in H&E. The complexity of microgranules in their cytoplasm becomes apparent in electron microscopy. Monocytes instead display a less dense kidney-shaped nucleus and dense cytoplasm. Eosinophils appear as bi-lobulated cells with a pink cytoplasm. In electron microscopy, we can observe larger granules. Basophils appear as mononucleated cells with a dark blue cytoplasm full of larger vesicles. Mast cells, which are rare in circulation, appear as dark blue cells in H&E with a complex network of granules in their cytoplasm. (b and c) Macrophages do not circulate per se and in tissues it is hard to distinguish them based on H&E alone. An exception is the mucosal macrophages in the lung, here exemplified by bronchiole macrophages, atop the columnar epithelia. (d) Multinucleated osteoclast. Electron micrographs show a complex cytoplasm, with prominent vacuoles in different stages of maturation and ample extracellular processes. These are representative images and apply to several species, including human, mouse, rat and rabbit.

surface molecules, as well as altered tissue components and apoptotic bodies. Miscellaneous phagocytic receptors include various scavenger receptors and lectin-like molecules such as Dectin-1, a myeloid phagocytic receptor for fungal ß-glucan and macrophage-restricted mannose receptors (MMR) that bind mannosyl and fucosyl residues on the surface of yeast, bacteria and parasites.

The process of phagocytosis is regulated by activation of various receptors such as the FcγRs initiating downstream signalling through immunoreceptor tyrosine-based activation motifs (ITAMs). The complement receptor CR3 recognises targets opsonised by C3bi, inducing cytoskeletal rearrangement due to actin polymerisation via Rho kinases. As a result of cross-linking, downstream effector functions are activated, resulting in phagocytosis, stimulation of the respiratory burst, degranulation of bactericidal proteins and activation of transcription factors, in turn leading to enhanced expression of genes encoding cytokines and other inducible proteins, discussed under inflammation. Defective uptake of pathogens and dying cells can lead to infection, autoimmunity and malignancy.

Adhesion and chemotaxis

Adhesion and chemotaxis are closely linked in phagocytes. The signals generated by chemical gradients activate the cytoskeleton of cells leading to propulsion and retraction of pseudopodia similar to those required for locomotion and adhesion. Locomotion is achieved by contraction of an actin filamentous network in the cortical gel at the leading front of the cell. Phagocytes possess a

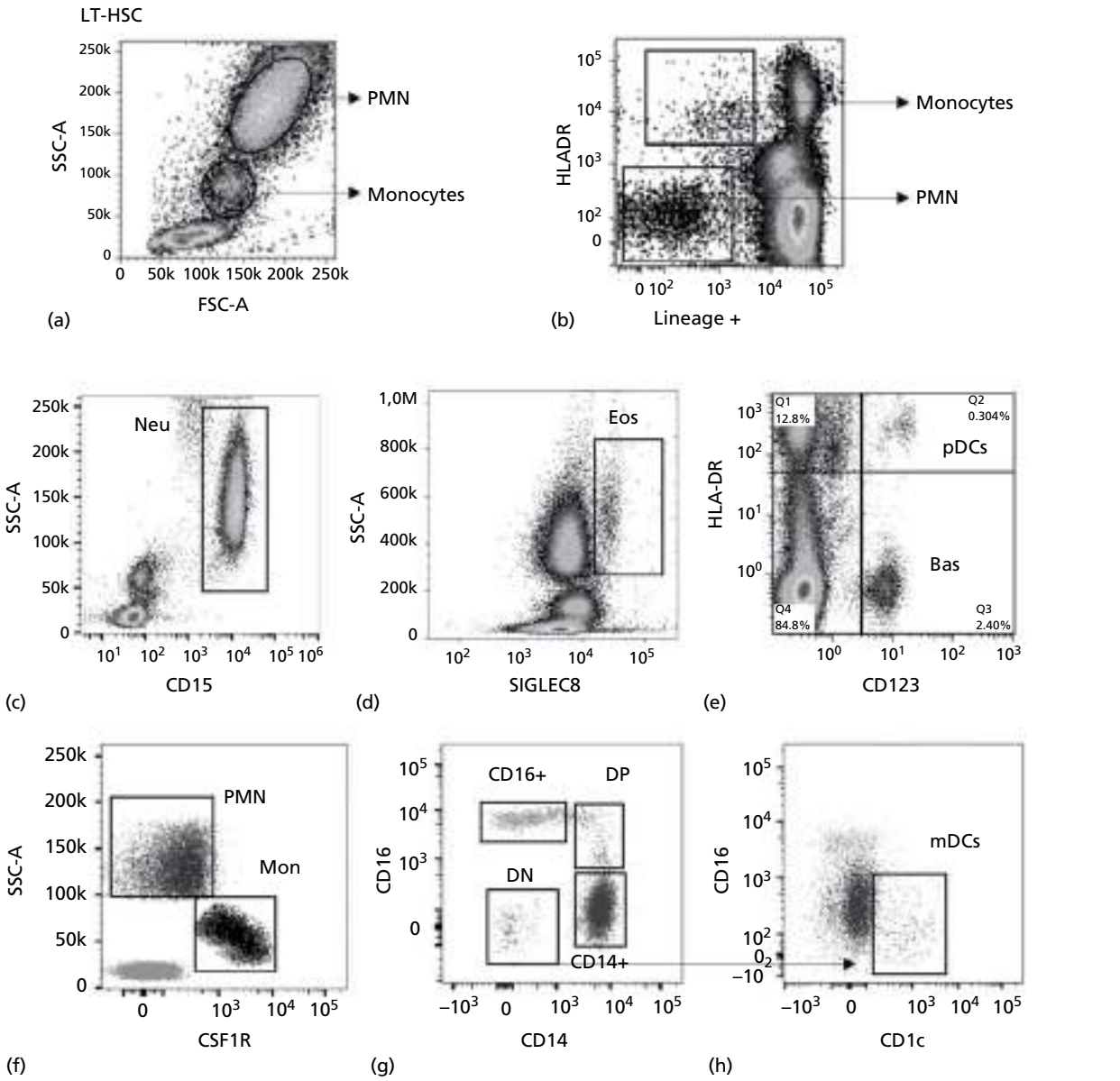

Figure 2.3 Origin and morphology of the circulating phagocyte family. (a) Scatter plot of white blood cells showing granulocytes, monocytes and lymphocytes. (b) All phagocytes are negative for lymphoid lineage cocktail markers containing anti-CD3, CD19, CD20 and CD56 antibodies. Further staining with HLADR shows MPS cells as positive and granulocytes negative. (c) CD15 stains neutrophils selectively. (d) SIGLEC 8 enables capture of eosinophils. (e) CD123 vs HLA-DR staining distinguishes HLADR-CD123+ basophils and HLADR+CD123+ plasmacytoid DCs. (f) MPS cells can be identified by CSF1R staining. (g) CSF1R+ cells can be further subdivided into 4 subsets based on CD14 and CD16 expression. (h) mDCs can be identified as CSF1R+ CD14-CD16-CD1c+ cells. DP, double positive; DN, double negative; mDC, myeloid DC.

number of cell–cell adhesion receptors and ligands, which mediate their recruitment, migration and interaction with other immune cells (Table 2.2). Phagocytes interact with the endothelium, slowly roll over and finally adhere firmly to endothelial cells (EC), to enter affected areas. Cells then spread their cytoplasm over the endothelium and crawl until they reach a suitable space to traverse the vessel wall, in a process called diapedesis. Diapedesis can be paracellular (between EC) or transcellular (across an EC), (Figure 2.4). Integrins (such as LFA1 and MAC1), immunoglobulin superfamily members (such as ICAM1 and VCAM1, which bind β_2 integrins) and selectins (such as L-, E- and P-selectins) mediate this process.

Table 2.1 Selected opsonic and non-opsonic receptors of phagocytes.

Receptor	Opsonic ligand and affinity	Cell type	Function
OPSONIC			
FcγRI (CD64)	IgG1-High (50 nmol/L)	Mon, Mac, Neu	Phagocytosis, respiratory burst
FcγRII (CD32)	IgG1 = IgG3 ≥ IgG4 = IgG2 Low (1 μmol/L)	Neu, Mon, Mac	Phagocytosis, respiratory burst
FcγRIII (CD16); FcγRIIIA (CD16a); FcγRIIIB (CD16b)	IgG1 = IgG3 Low (110 nmol/L); Low (470 nmol/L)	Neu, Mon, Mac	Phagocytosis
FcαR (CD89)	IgA1, IgA2, secretory IgA1 and IgA2	Neu, Mon, Mac	Phagocytosis, respiratory burst, bacterial killing
CR1 (CD35)	C3b and C4b dimers	Neu, Mon, Mac,	Phagocytosis
CR3 (CD11b/CD18)	C3bi	Neu, Mon, Mac,	Phagocytosis, respiratory burst
NON-OPSONIC			
Dectin 1	ß -glucan	DCs, Mon, Mac, Neu	Phagocytosis
Mincle	Dimycolate	DCs, Mon, Mac, Neu	Phagocytosis
MCL	α-mannan	DCs, Mon, Mac, Neu	Phagocytosis
DC SIGN	Fucosylated and Mannosylated ligands	DCs, Mon, Mac	Phagocytosis
CD14	LPS	Mon, Mac, Neu	Lipid recognition
Scavenger Receptor A	LPS, polyanionic, apolipoproteins A1/E, oxidised LDL	Mac	Phagocytosis, endocytosis, adhesion
CD36	PS	Mac, Mon, ECs, adipocytes, thrombocytes, megakaryocytes, skeletal and cardiac muscle	Phagocytosis, fusion
MARCO	Trehalose Dimycolate, LPS	Mac, DCs	Phagocytosis, innate activation
Mannose receptor (CD206)	Mannosylated proteins (mannosyl-, fucosyl- or GlcNAc ligands)	Mac, immature DCs	Phagocytosis, endocytosis
CD163	Haemoglobin-haptoglobin complexes	Mac, Mon, DCs	Phagocytosis, endocytosis

Integrins recognise extracellular and intracellular stimuli, mediating 'outside-in' and 'inside-out' signalling. The affinity of integrins for extracellular ligands, such as ICAM-1 or fibrinogen, is greatly increased by inside-out signalling. Inside-out signalling is activated by mechanical stress and plays a crucial role in neutrophil arrest during chemotaxis. In fact, during phagocyte rolling on endothelial cells, β2 integrins are progressively stretched, increasing inside-out signalling and culminating in high-affinity adherence to endothelium. The β_2 integrin family consists of three leucocyte-restricted integrins, LFA-1 (CD11a/CD18), CR3 (MAC-1, CD11b/CD18) and p150/95 (CD11c/CD18). Mutations causing defects in the expression and function of β2 integrins or selectins increase susceptibility to infection, through defective migration of neutrophils to infected sites.

Selectins are expressed on all leucocytes (L-selectins) as well as postcapillary endothelial surfaces (E-selectins) and in platelet α-granules and endothelial cell Weibel–Palade bodies (P-selectins). The interaction of E- and P-selectins on cytokine-activated endothelial cells, and L-selectins, targets phagocytic cells to the endothelium at sites of vascular injury and initiates the rolling movement of leucocytes along the vessel wall. In addition, a newly characterised leucocyte family of seven transmembrane G protein-coupled adhesion receptors (GPCRs) contains several members implicated in myeloid phagocyte adhesion, differentiation and activation.

After diapedesis phagocytes migrate directionally in response to spatial gradients of chemical substances in a process known as chemotaxis. Chemotactic stimuli include microbial products and inflammatory stimuli secreted by cells. Chemotactic GPCRs include chemokine receptors such as CXCR1, 2 and CXCR4, which bind CXC chemokines in PMN and CCR2 and CCR1 which bind CC chemokines in MPS cells (Table 2.3). Non-chemokine chemotactic receptors include the

Table 2.2 Phagocyte adhesion molecules.

Adhesion molecule	CD number	Cellular distribution	Ligand	Function
Integrin family				
Very late-acting antigens				
αβ (VLA-1)	CD49a/29	Mon, EC	Collagen I, IV, laminin	Cell adherence to ECM
$\alpha_2 \beta_1$ (VLA-2)	CD49b/29	Mon, EC, platelets	Collagen I, IV, laminin	Recruitment to extravascular tissue
$\alpha_3 \beta_1$ (VLA-3)	CD49c/29	Mon	Collagen I, laminin, fibronectin	Cell adherence to ECM
$\alpha_4 \beta_1$ (VLA-4)	CD49d/29	Mon, eos, bas	Fibronectin, VCAM-1	Cell adherence to ECM and cell–cell adhesion matrix
$\alpha_5 \beta_1$ (VLA-5)	CD49e/29	Mon, neu, EC	Fibronectin	Cell adherence to ECM
$\alpha_6 \beta_1$ (VLA-6)	CD49f/29	Mon	Laminin	Cell adherence to ECM
Leucocyte integrins (LFA-1 family)				
$\alpha_D \beta_2$	–/18	Mac	ICAM3, VCAM-1	Cell adherence to ECM
$\alpha_L \beta_2$ (LFA-1)	CD11a/18	Mon, Mac, granulocytes	ICAM-1, ICAM-2, ICAM-3	Cell–cell adhesion and cell–matrix adhesion
$\alpha_M \beta_2$ (CR3, Mac-1)	CD11b/18	Mon, Mac, granulocytes	ICAM-1, C3bi, fibronectin, factor X, microbial antigens	Endothelium adherence/ extravasation
$\alpha_X \beta_2$ (p150,95)	CD11c/18	Mon, Mac, granulocytes	C3bi, fibronectin	Adhesion during inflammatory response
Cytoadhesins $\alpha_V \beta_3$ (vitronectin receptor)	CD51/61	Mon, EC	Vitronectin, fibronectin, collagen, thrombospondin, vWF	Cell adherence to ECM
$\alpha_R \beta_3$ (leucocyte response integrin)		Mon, granulocytes	Vitronectin, fibronectin, collagen, thrombospondin, vWF	Cell adherence to ECM
$\alpha_V \beta_5$	CD51/–	Mon	Vitronectin, fibronectin	Cell adherence to ECM
$\alpha_V \beta_7$	CD51/–	Mac	?	
Immunoglobulin superfamily				
ICAM-1	CD54	Mon, EC	$\alpha_L \beta_2$, $\alpha_M \beta_2$	Cell–cell adhesion
ICAM-2	CD102	Mon, EC	αβ	Cell–cell adhesion
ICAM-3	CD50	Mon, granulocytes	$\alpha_{L2} \alpha_L \beta_2$	Cell–cell adhesion
VCAM-1	CD106	Mac, EC, dendritic cells	$\alpha_4 \beta_1$	Recruitment
PECAM-1	CD31	Mon, EC, platelets	CD31, $\alpha_V \beta_3$	Transmigration
HCAM	CD44	Ubiquitous	Collagen I, IV, fibronectin	Extravasation
Selectin family				
L-selectin	CD62L	Mon, granulocytes	Carbohydrate determinants on EC	Migration, rolling on vessel wall
E-selectin	CD62E	Neutrophil, EC	Mo, Neu, Eos	Migration, rolling on vessel wall
P-selectin	CD62P	EC, platelets	Mo, Neu, Eos	Adhesion to activated platelets and EC

Bas, basophil; CD, cluster of differentiation; EC, endothelial cell; Eos: eosinophil; ICAM, intercellular adhesion molecule; Mac, macrophage; Mon, monocyte; Neu, neutrophil.

leukotriene B_4 receptor BLT1 and platelet-activating factor (PAF) receptor and formylated peptide receptors 1 and 2, recognising not only formylated peptides, but also other products from bacteria or mitochondria and finally complement receptors such as the C5a receptor. All three major GPCR-activated pathways (i.e. the PLCβ, PI3K and Src pathways) converge in triggering phagocyte responses, including superoxide production, degranulation and chemotaxis.

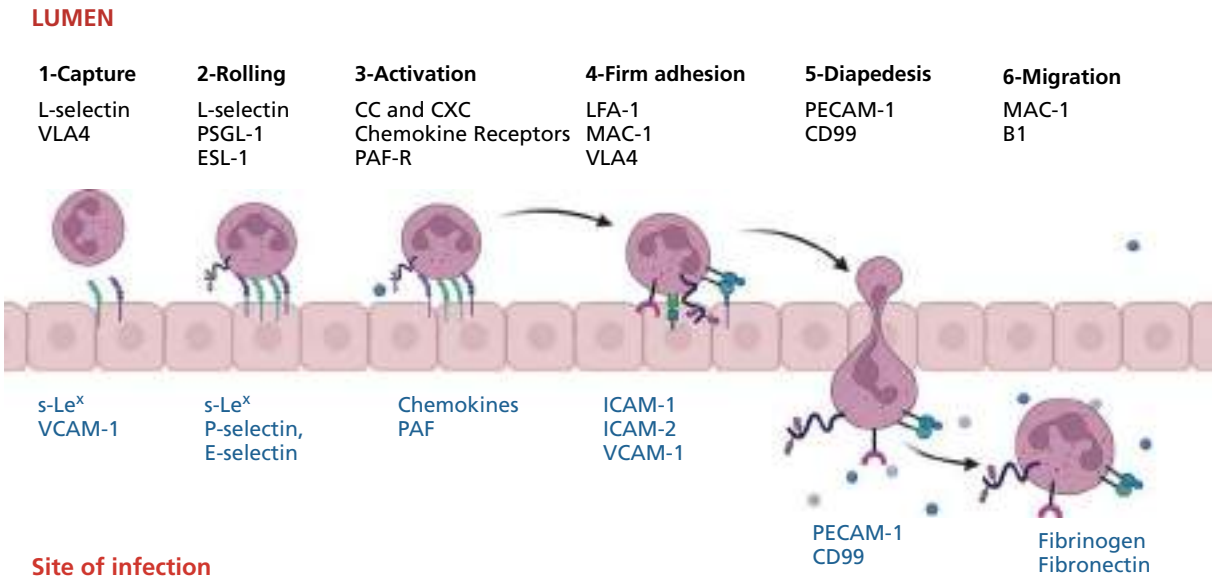

Figure 2.4 Diapedesis and chemotaxis. In inflammation, phagocytes migrate towards the location of the specific stimulus. The process starts with rolling of the leucocytes to the wall vessel. Paracellular diapedesis is the purposeful coordinated movement of leucocytes though the endothelial lining towards sites of inflammation. Selectin-mediated leucocyte tethering and rolling across endothelial surfaces is followed by integrin-mediated firm adhesion and leucocyte polarisation at endothelial cell junctions. With increased contact provided by adhesion molecules, the interaction between leucocytes and endothelial cells increases, leading to rolling of the cells. Increased integrin activation supports firm adhesion and enables transmigration. We present the main steps, and adhesion molecules involved. Phagocyte molecules appear in black, and endothelial cell or ECM molecules in blue.

Table 2.3 Examples of Chemotactic factors controlling phagocytes.

Chemokine receptors	Ligands	Cells
CC receptors, CCR1, CCR2, CCR5, CCR7	CC chemokines, CCL2, CCL3, CCL4	Mainly monocytes and DCs
CXC Receptors CXCR1, CXCR2, CXCR4	CXC Chemokines, CXCL1, CXCL2, CXCl8	Mainly granulocytes
Complement receptors		
C5aR	C5a complement component	All phagocytes
C3aR	C3a complement component	All phagocytes
Formyl peptide receptors		
FPR1, FPR2	High and low affinity for Bacteria and Mitochondria – derived formyl peptides, Lipoxin A4	All phagocytes
Lipid receptors		*All phagocytes*
BLT1 and BLT2	Leukotriene B4 high-affinity receptor BLT1 or the low-affinity receptor BLT2	All phagocytes

Pattern recognition receptors and inflammation

Phagocytes efficiently detect the presence of microbes or cell damage whilst patrolling tissues. This is accomplished via Pattern recognition receptors (PRRs), which bind selectively to evolutionary conserved microbial molecules, pathogen-derived molecular patterns (PAMPS), or to products expressed or released by damaged cells, damage-derived molecular patterns (DAMPS). Important PRRs in phagocytes include the membrane Toll-like receptors and C-type lectin receptors, and cytosolic receptors such as Nod-like receptors and AIM2. TLRs are the best-studied PRR in phagocytes. TLR activation triggers signalling pathways involving MyD88 and MAP kinases, and activation of the transcription

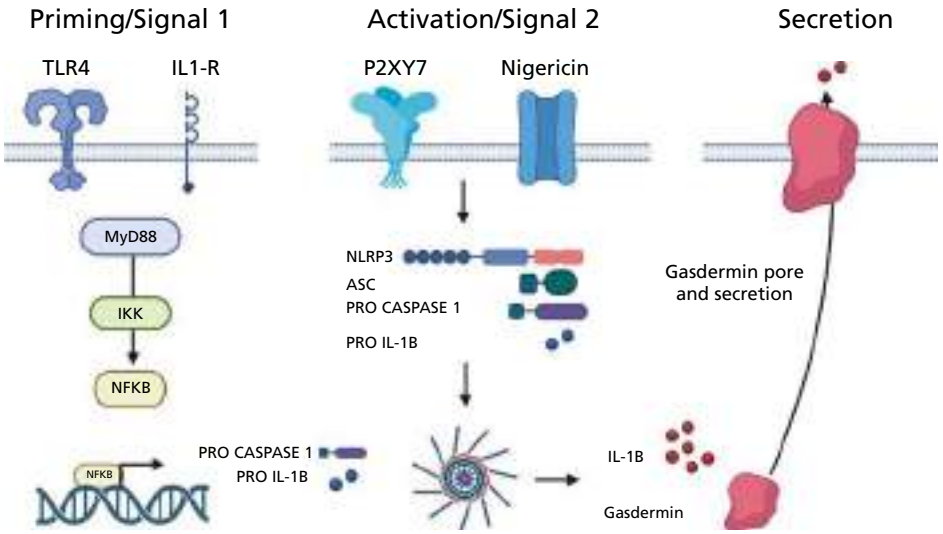

Figure 2.5 Priming and activation of the NLRP3 inflammasome. Phagocytes require two signals to produce and secrete IL-1β and IL-18. The first step is priming, provided by LPS recognised by TLR4, which leads to activation of NF-κB and synthesis of pro-IL-1β and pro-IL-18. NLRP3 expression itself can be regulated and increased in this reaction. The second signal involves cell damage sensed in a variety of forms such as extracellular ATP, presence of intracellular bacteria or insoluble crystals. These signals activate pattern receptors such as NLRP3. The ASC adaptor amplifies the response and activates pro-caspase 1. Inflammatory caspases cleave gasdermin, which forms pores and leads to pyroptosis (Source: Groslambert and Py 2018; Zhang *et al*. 2020.)

factors NF-κB and AP-1, which lead to the production of proinflammatory cytokines, and TRIF-associated IRF responses that activate interferon production. The cascades alter the transcriptome of the cells and result in increased secretion of inflammatory products and enzymes.

Secretion is a specialty of phagocytes. TNF is secreted from two pathways directly or by shedding action of Adam17. IL-10 and IL-6 are secreted directly into secretory vesicles. Another important mechanism is inflammasome activation which leads to secretion of IL-1β and IL-18. IL-1β and IL-18 are synthesised as a proproteins and activated by caspases in the cytosol. Nod-like receptors such as NLRP3 or sensors of aberrant RNA or DNA, promote assembly of multiprotein complexes, called inflammasomes, which further lead to caspase activation (Figure 2.5). Caspases cleave the pro forms of IL-1β and IL-18. Gasdermin, a membrane lytic agent implicated in IL-β release, is a product of DNA sensing and activation of the STING pathway. TLR activation also increases enzymes that process cellular lipids into inflammatory or anti-inflammatory products, including prostaglandins, leukotrienes, lipoxins and maresins.

Killing via lytic enzymes and production of reactive radicals

The ultimate purpose of phagocytosis is killing microbes. Several mechanisms lead to bulk product release by neutrophils, monocytes and macrophages, e.g. lysozyme acting on muramyl peptidoglycan in bacterial walls. Neutral proteinases initiate plasma protein cascades involved in coagulation, fibrinolysis, complement activation and generation of kinins and angiotensin to control infection. Neutrophil granules are secreted by a process called degranulation. Some granules contain receptors that can be mobilised to the cell surface and enable neutrophils to respond rapidly to environmental cues. Other molecules contained in the granules, such as leukotriene B4, function as secondary chemoattractants. Neutrophils contain four types of granules that are produced sequentially during their maturation and can be identified by marker enzymes or proteins (Table 2.4). In addition, certain vacuoles that are part of the endosomal system, such as multivesicular bodies, can be used by neutrophils to deliver various products extracellularly by a process called reverse endocytosis. We discuss neutrophil granules in detail in the next section.

Monocytes have rudimentary granules and can be induced to synthesise and secrete a variety of products *de novo*. They instead carry their antimicrobial peptides and digestive enzymes in the lysosome. Lysosomes are organelles with specific membrane proteins, a complex biogenesis, and contain over 50 lytic enzymes that can be secreted into the phagosome or the extracellular space. Many of these enzymes underlie phagocyte disorders. Upon phagocytosis, pathogens in phagosomes are killed by the release of granules and specialised lysosomal content. For example, many of the proteins stored in PMN granules display antimicrobial activities,

Table 2.4 Neutrophil microbicidal proteins.

Protein	Characteristics	Target organisms	Effects on target
Non-enzymatic proteins			
BPI	Highly cationic, neutralises LPS, most potent microbicidal protein, not released from granule	Gram-negative bacteria	Binds lipid A region of LPS, increases bacterial membrane permeability, activates bacterial degradative enzymes
Defensins	Comprise 30–50% of azurophil granule protein	Gram-positive > Gram-negative bacteria, fungi, viruses, mammalian cells	Increases membrane permeability
Lactoferrin	Cationic, stimulates hydroxyl radical formation	Gram-positive and Gram-negative bacteria, fungi	Oxidative damage
Catalytic proteins and analogues			
Proteinase 3	Serine proteinase	Escherichia coli, Streptococcus faecalis, Candida albicans	Growth inhibition
Cathepsin G	Serine proteinase	Gram-positive and Gram-negative bacteria, fungi	Inhibition of peptidoglycan synthesis
Azurocidin	Serine proteinase	Gram-negative bacteria	Non-catalytic mechanisms
Lysozyme	Cationic	Gram-negative bacteria, few Gram-positive bacteria	Potentiation of complement and H_2O_2 killing, cleavage of cell wall peptidoglycans
Elastase	No direct microbicidal activity		Coactive with lysozyme, potentiation of MPO–halide–H_2O_2 system

BPI, bacterial permeability-inducing factor; LPS, lipopolysaccharide; MPO, myeloperoxidase.

summarised in Table 2.4. After phagocytosis, the granule contents are released into the phagosome resulting in bacterial killing.

The respiratory burst generates superoxide anion (O_2^-), the highly reactive hydroxyl radical ($OH^\cdot$) and other reactive oxygen species (ROS). These oxidative products kill microbes both directly and indirectly, possibly through their ability to activate metalloproteinases such as elastase and collagenase. Production of (O_2^-) results from activation of NADPH oxidase, a multicomponent enzyme with several subunits located in various regions of the quiescent phagocytes (Figure 2.6).

Generation of reactive nitrogen intermediates through nitric oxide synthase (NOS) is another mechanism for phagocytic killing of microbes. Nitric oxide (NO) is the highly reactive free radical product of the oxidation of L-arginine in phagocytes during inflammation. Three distinct isoforms of NOS have been described in blood cells. Two are found in endothelial and neuronal cells and the third, inducible NOS, is induced by cytokines, such as IFN-γ, in a number of cell types. NO has an important role in the antimicrobial activity of both neutrophils and activated mononuclear phagocytes. NOS is located in the cytoplasm and mediates defence against facultative intracellular pathogens, as well as other prokaryotic and eukaryotic pathogens.

Phagocyte particulars

Neutrophils

Neutrophils are the first leucocytes to be recruited to inflammatory sites and their rapid deployment is essential to clear infection. They are essential for life and neutropenia brings a serious risk of fatal infection. The term *neutrophil* derives from the staining characteristics of their specific granules. Neutrophils are also referred to as *polymorphonuclear* (Greek, 'having a nucleus with various shapes') leucocytes owing to the characteristic lobular nucleus that is segmented into two to five lobes connected by thin chromatin strands. A curved, but not lobular, nucleus is present in band neutrophils, an immature cell type in transition to segmented neutrophils, (Figure 2.7). Very few or no band cells are normally detected in the blood, although their number may increase during severe infections. Neutrophils have a diameter of 8–10 μ in circulation.

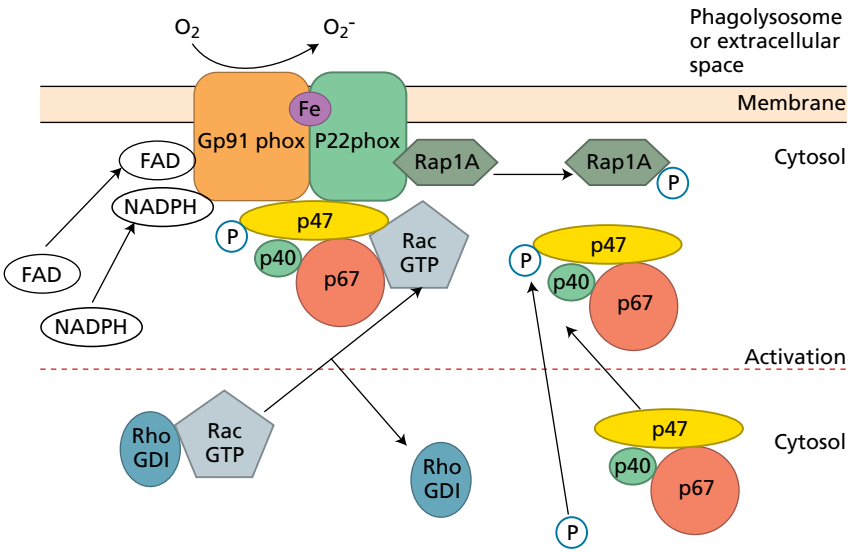

Figure 2.6 Components of the NADPH oxidase system. The components include a 47-kDa cytosolic protein (p47), a 67-kDa cytosolic protein (p67), a 40-kDa cytosolic protein (p40), cytosolic G-proteins (Rac and Rap1) and a membrane-bound cytochrome (b_{558}). The cytochrome consists of haem-containing p22-phox and gp91-phox. The gp91 subunit is a FAD-dependent flavoprotein shuttling electrons to molecular oxygen, forming O_2^-. The p47 component can be phosphorylated to various extents. In activated cells, the p40, p47 and p67 proteins translocate to the membrane to form an activation complex with cytochrome b_{558}. Similarly, the Rac and Rap1 proteins also translocate. The activated oxidase passes electrons from NADPH via FAD to oxygen, thereby generating superoxide.

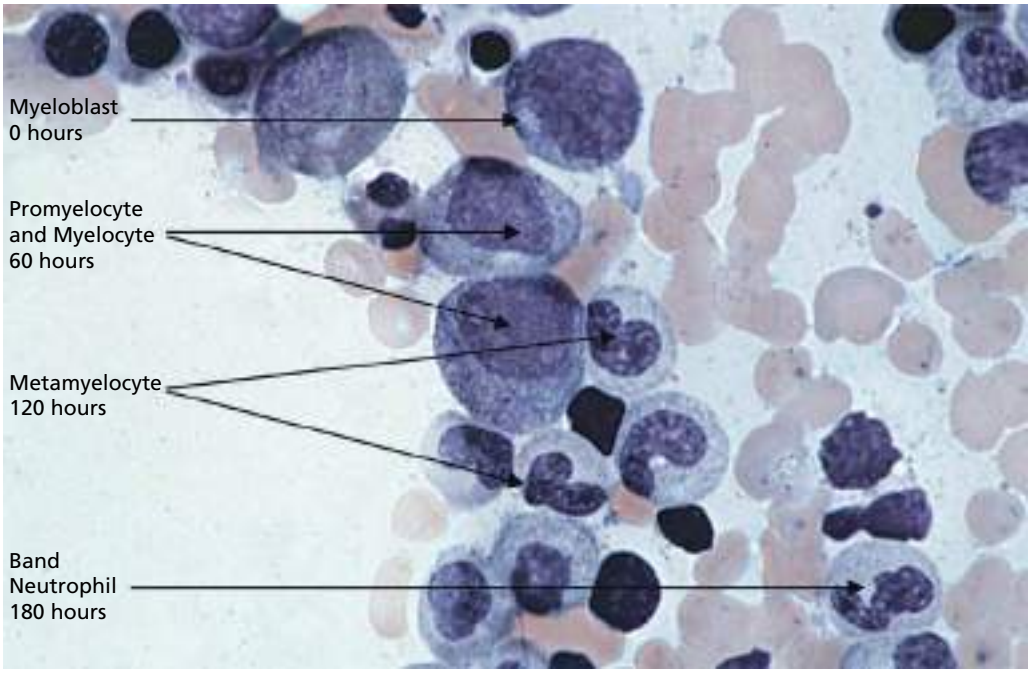

Figure 2.7 Stages of neutrophil maturation. Stages of neutrophil maturation, showing a myeloblast, a promyelocyte, several myelocytes and metamyelocytes, a band cell and a segmented neutrophil in a bone marrow aspirate.

In bone marrow, neutrophil-committed progenitors can be divided into neutrophil progenitors (Lin-CD34+/-CD86-CD15+ CD66b+) and pre-neutrophils (Lin-CD34-CD101-CD15+ CD66b+). Pre-neutrophils differentiate into immature neutrophils, which make up the post-mitotic pool and consist of metamyelocytes (kidney-shaped nucleus and light cytoplasm) and band cells (both Lin-CD34-CD101+CD15+CD66b+ CD10-CD16-). The bone marrow also contains a large pool of mature neutrophils (CD101+CD15+CD66b+ CD10+CD16+) that can be readily released into the circulation.

Neutrophil maturation is orchestrated by several transcription factors, such as PU.1, growth factor independence 1 (GFI1), and members of the CCAAT/enhancer binding protein family (C/EBPα-ζ). As previously mentioned, CSF3 or G-CSF is a crucial mediator of neutrophilic granulopoiesis that also induces the release of mature granulocytes from the bone marrow into the circulation and is used as a drug to increase neutrophil production in patients with neutropenia. G-CSF is produced through a cytokine circuit initiated by macrophage-derived IL-23, which triggers IL-17 release from γδ T cells and the ILC_3 subset of innate lymphoid cells. In turn, IL-17A induces G-CSF production by bone marrow stromal cells, macrophages and endothelial cells. Notably, the production of IL-23-dependent IL-17A is greatly increased by the proinflammatory cytokines IL-1β or IL-18, which contribute, by this mechanism, to boost neutrophil production during acute inflammation and infection.

Retention of mature neutrophils in the bone marrow and release in the circulation are governed by two opposing chemokine/chemokine receptor systems (respectively, CXCL12/CXCR4 and CXCL8/CXCR2). G-CSF increases the expression of CXCR2 and downregulates CXCR4 on neutrophils and HSC, thereby inducing the release of neutrophils into the bloodstream and migration of HSC to the spleen, liver and inflamed tissues under emergency granulopoiesis. The G-CSF-chemokine axis can also be exploited to harvest HSCs for therapeutic purposes. For example, coadministration of G-CSF and a CXCR4 antagonist effectively mobilises HSCs from the bone marrow into the peripheral blood.

In performing their defensive functions, neutrophils can produce considerable collateral damage to host tissues, particularly by releasing their contents extracellularly. Azurophilic granules, otherwise known as primary granules, are produced first and are present in promyelocytes and all further stages of neutrophil differentiation (Table 2.5). They contain microbicidal proteins, including the protease azurocidin, and acid hydrolases (e.g. myeloperoxidase and lysozyme) involved in oxidative and non-oxidative killing of bacteria and fungi. Azurophilic granules also contain various amounts of defensins and can be classified in humans into $defensin^{high}$ and $defensin^{low}$ granules.

Specific or secondary granules are smaller than azurophilic granules and contain lactoferrin, cysteine-rich secretory protein 3, ficolin 1 and haptoglobin as well as chemotactic, opsonic and adhesion protein receptors. The release of haptoglobin from specific granules after neutrophil activation at sites of inflammation may reduce local tissue injury. Tertiary granules contain matrix metalloproteinase 9, also known as gelatinase B, and are therefore also called gelatinase granules. Preformed proteins, including activation receptors, are present in secretory vesicles, which can be rapidly mobilised to the cell surface, where their contents are incorporated in the plasma membrane. The presence of multiple kinds of granules can be explained by the need of neutrophils to release different proteins at different times during their activation process.

Neutrophils are short-lived after entering the bloodstream. Their exact lifetime in humans can be estimated at 36 hours (18 hours each in the circulation and in peripheral organs, where they die). In the steady state, neutrophils patrol organs (e.g. the liver, spleen and lung) and body surfaces (e.g. the skin, mucosa and mucus layers) to detect the presence of microbial products or tissue damage. Comparatively few neutrophils enter the central nervous system and the gonads. The lifespan of neutrophils in tissues can be extended up to tenfold by microbial products and proinflammatory mediators, including G-CSF, GM-CSF, IL-8, C-reactive protein and serum amyloid A. Whilst migrating through the vessel wall neutrophils produce factors such as IL-8 that act as secondary attractants. This relay of signals between neutrophils choreographs dynamic cell aggregation in a process called 'swarming' (Figure 2.8), which can be visualised by intravital microscopy and extends the recruitment range of neutrophils over long distances.

Efferocytosis of recruited neutrophils is essential to resolve inflammation and repair damaged tissues. In contrast, lytic forms of cell death, such as necroptosis, pyroptosis and NETosis (see below) often occur during various pathological conditions, resulting in amplification of the inflammatory response and in potential tissue injury. At least one billion neutrophils per kg of body weight undergo efferocytosis every day in various organs, especially the bone marrow, followed by the liver and spleen. Thus, the bone marrow is both the main haematopoietic organ and the dominant site of neutrophil efferocytosis. The latter process attenuates granulopoiesis in a negative feedback loop involving G-CSF-mediated regulation.

Neutrophils are described as 'quiescent', 'primed' or 'fully activated'. Neutrophils circulate in the quiescent state, thus limiting potential damage to vascular walls. Various agonists are responsible for priming and activating neutrophils, including contact with other cells, chemotaxins, antigen-antibody complexes, complement components, inflammatory cytokines, growth factors, microbial components and products of damaged tissues. In an extreme form of activation, neutrophils release their nuclear material extracellularly. This results

Table 2.5 Neutrophil granules and their contents.

Granule	Azurophilic (primary)	Specific (secondary)	Gelatinase (tertiary)	Secretory vesicles
Marker enzyme	Myeloperoxidase	Lactoferrin	Gelatinase	Alkaline phosphatase
Membrane	CD63, granulophysin, CD68, V-type H^+ -ATPase	CD15, CD66, CD67, CD11b/CD18, cytochrome b, fMLP-R, fibronectin-R, G-protein α-subunit, laminin-R, NB-1 antigen, 19-kDa protein, 155-kDa protein, Rap-1, Rap-2, SCAMP, thrombospondin-R, TNF-R, urokinase-type plasminogen activator-R, VAMP-2, vitronectin	CD11b/CD18, cytochrome b, diacylglyceroldeacylating enzyme, fMLP-R, SCAMP, urokinase-type plasminogen activator-R, VAMP-2, V-type H+ -ATPase	CD10, CD13, CD45, CD14, CD16, CD35 (CR1), CD11b/CD18, alkaline phosphatase, fMLP-R, SCAMP, urokinase-type plasminogen activator-R, V-type H+ -ATPase, VAMP-2, C1q-receptor, decay activating factor
Matrix, microbicidal	Myeloperoxidase, nitric oxide synthase, lysozyme, BPI protein, defensins, serpocidins, elastase, cathepsins, proteinase 3, azurocidin (CAP 37)	Lactoferrin, lysozyme	Lysozyme	
Matrix, hydrolases	Acid β-glycerophosphatase, α-mannosidase, β-glucuronidase, β-glycerophosphatase, N-acetyl-βglucosaminidase, sialidase	Gelatinase, collagenase, histaminase, heparanase, NGAL, sialidase	Gelatinase, acetyltransferase	
Matrix, other	Acid-mucopolysaccharide, heparin-binding protein	β2-Microglobulin, urokinase-type plasminogen activator, vitamin B12-binding protein	β2-Microglobulin,	Plasma proteins (including tetranectin)

fMLP, f-Met-Leu-Phe; NGAL, Neutrophil gelatinase-associated lipocalin; R, receptor; SCAMP, secretory carrier membrane protein; VAMP, vesicle-associated membrane protein.

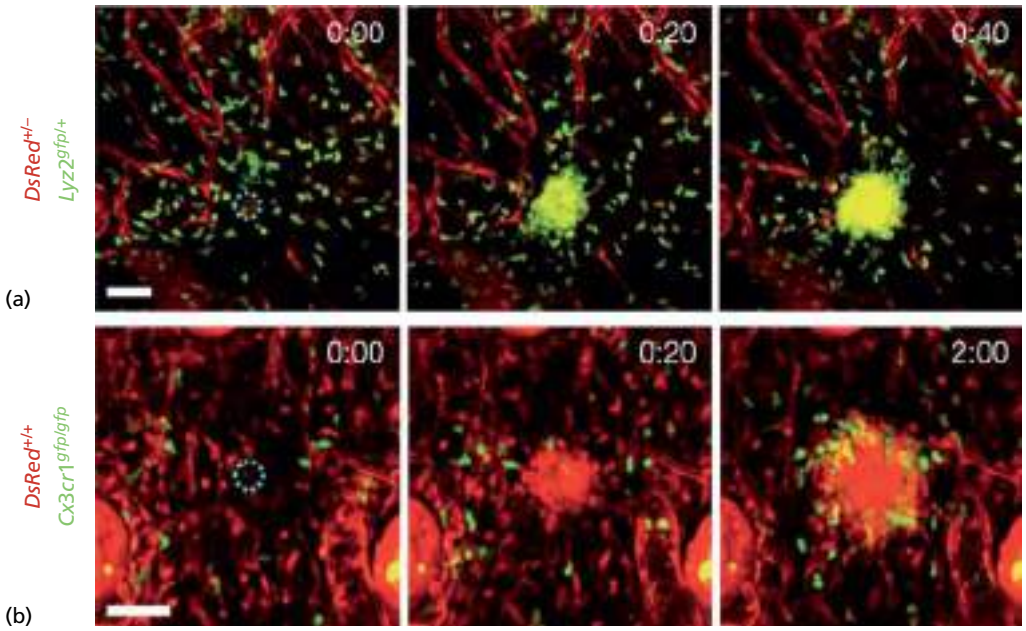

Figure 2.8 Neutrophil and monocyte swarming. The recruitment of neutrophils and monocytes to injury sites is incremental and comparable to swarming. Cells that have infiltrated leave an activation trail that other cells follow. (a) Shows intravital microscopy on an injury performed in the ear dermis of anaesthetised mice. A time-lapse shows infiltration of yellow and green Lyz2gfp/+ myelomonocytic cells, into red stroma. (b) Shows infiltration of Cx3cr1gfp macrophages and monocytes in yellow–green, and neutrophils and stroma in red. Scale bars, 50 μm. Time, h:min (Lämmermann *et al*. 2013).

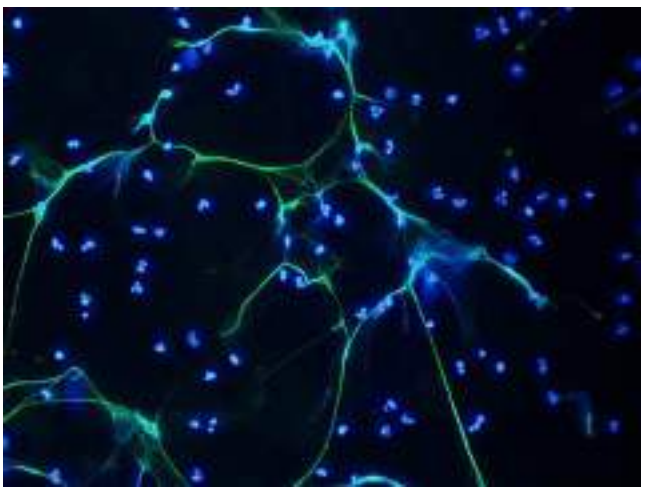

Figure 2.9 Neutrophils produce DNA extracellular traps. Representative images of NETs induced in vitro by LPS in human neutrophils. NETs are visualised by co-staining of neutrophil elastase (green) and nuclear material (DAPI, blue). Original magnification ×40 (Kaplan and Radic 2012).

in the formation of 'neutrophil extracellular traps' (NETs) consisting in core DNA and histone filaments decorated with antimicrobial proteins and enzymes (Figure 2.9). NETs trap microorganisms allowing their phagocytosis by other neutrophils and macrophages. This process often triggers a form of programmed cell death called NETosis.

Eosinophils, basophils and mast cells

Eosinophils, basophils and mast cells are the less abundant, yet powerful granulocytes. The three are specialised in defence against nematodes and other parasites and are implicated in allergic responses. The eosinophil precursors are positive for IL-5Rα+CD34+CD38+ and can be found in bone marrow, adult and cord blood. IL-5, IL-3 or GM-CSF promote their maturation. The eosinophil precursors expand in eosinophilic patients. Basophils are produced from IL-5Rα−CD34+CD38+ progenitors. Eosinophils and basophils go through promyelocyte, myelocyte and metamyelocyte stages. Mast cell progenitors migrate from the bone marrow, homing to tissues where they mature. Mast cell progenitors instead, express the stem cell factor (SCF) receptor c-kit and the high-affinity IgE receptor FcεRI.

In the steady state, eosinophils are present in a variety of tissues including the lung, thymus, adipose tissue and gastrointestinal tract to mention a few. As for PMN several activation stages have been suggested. There are three distinct eosinophilic granules, the round uniformly electron-dense primary granules present mainly in the eosinophilic promyelocyte/myelocyte stages, secondary or specific granules, and finally less well-characterised small granules (Table 2.6).

Table 2.6 Contents of eosinophil granules.

Granule	Protein content	Comment
Primary granule (Charcot–Leyden granule)	Charcot–Leyden crystal (CLC) protein	Weak lysophospholipase, carbohydrate-crystal protein-containing binding properties
Specific granule (secondary granule)	Eosinophil peroxidase Major basic protein (MBP) Eosinophil cationic protein (ECP)	Toxic to parasites Ribonuclease, bactericidal, toxic to parasites
	Eosinophil-derived neurotoxin (EDN)	Ribonuclease, toxic to parasites
	Eosinophil peroxidase (EPO)	Antibacterial
	Lysophospholipase	
	Acid phosphatase	
	Arylsulfatase B	
	Phospholipase A_2 (secretory)	Antibacterial
	BPI protein	LPS binding, antibacterial
	NAMLAA	Antibacterial
	FAD	
	Catalase	
	Urokinase	
	CD63	Tetraspanin
	Proteoglycan	
	α_1 -Antitrypsin	
Small-type granule	Arylsulfatase B	Lysosomal hydrolase
Secretory vesicle	Cytochrome b_{558}	NADPH oxidase component
	CR3	β_2 integrin

BPI, bactericidal permeability-increasing protein; FAD, flavin adenine dinucleotide; LPS, lipopolysaccharide; NAMLAA, *N*-acetylmuramyl-L-alanine amidase.

Primary granules contain eosinophil peroxidase and Charcot–Leyden crystal protein. The eosinophil peroxidase is distinct from neutrophil MPO and can mediate damage to microorganisms, host tissues and induce bronchoconstriction in asthma. The large specific granules contain the eosinophil's cytotoxic and proinflammatory proteins and account for more than 95% of granules in the mature eosinophils, conferring the characteristic appearance of the cell. Eosinophil granules contain a number of enzymes similar to those found in the primary lysosomes of the neutrophil, but eosinophils lack lysozyme. Different cationic polypeptides are the major constituents of eosinophil granules and include major basic protein (MBP), eosinophil cationic proteins (ECP) and eosinophil-derived neurotoxin (EDN).

Eosinophils are recruited in response to early- and late-phase components of immediate hypersensitivity reactions. Their activation and recruitment involve the interaction of several adhesion pathways and chemotactic agents, including the complement fragment C5a, PAF, IL-3, IL-5, GM-CSF, IL-2, RANTES, eotaxins and the CD8+ T-cell-derived lymphocyte chemoattractant factor (LCF). The specificity and intensity of the microbicidal function of eosinophils differ from those of other leucocytes. Eosinophils preferentially assemble NADPH oxidase in the plasma membrane to generate extracellular O_2–, (Figure 2.10). The major role of eosinophils in host defence is the destruction of metazoan parasites. Eosinophils readily degranulate in response to stimulation by antigens, cytokines and complexed or secretory IgA, IgE and IgG (Hirsch 2022). Proteins released from eosinophils result in histamine release from basophils and mast cells and amplify the inflammatory response (Table 2.7). These proteins are also powerful toxins towards host cells, leading to tissue injury.

Basophils and mast cells

Basophils and mast cells express the receptor that binds with high affinity to the Fc portion of IgE antibodies (FcεIR) and have large metachromatic (purple–black) granules rich in histamine, serotonin and leukotrienes. Basophils have a bilobed nucleus, in contrast to mast cells, which have a unilobed nucleus. More recent studies have demonstrated that despite their significant similarities, basophils and mast cells are terminally differentiated progeny of distinct bone marrow progenitors. Basophils mature in the marrow over a period of 2–7 days and after their release into the

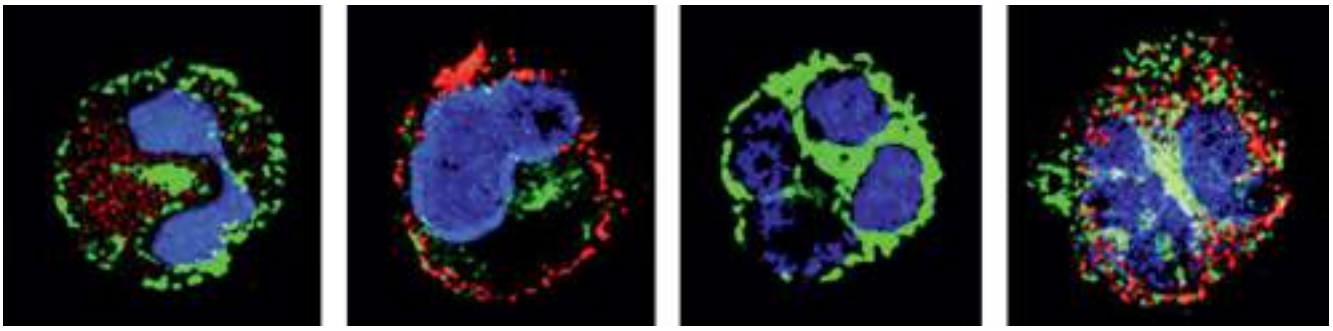

Figure 2.10 The respiratory burst in eosinophils and neutrophils is assembled in different locations. Eosinophils are specialised in dealing with extracellular pathogens. Rac GTPase (red) binds GTP to stimulate NADPH oxidase activity p22phox (green) and becomes associated with their plasma membrane upon cell activation. Nuclei are shown in blue. Resting eosinophils shown in (a–c), versus PMA-stimulated eosinophil in (d), show that p22phox and Rac colocalise in the cell membrane. In neutrophils the complex forms intra-cellularly. Original magnification, ×40 (Lacy *et al.* 2003).

Table 2.7 Biologic activities of selected Eosinophil granule proteins.

Granule protein	Biologic activities
Major Basic Protein (MBP)	Helminthotoxin and cytotoxin; bactericidal, platelet agonist; causes histamine release from basophils and rat mast cells; neutralises heparin; increases bronchial reactivity to methacholine; provokes bronchospasm; activates neutrophils
Major Basic Protein-2 (MBP-2)	Cytotoxin; causes histamine release and LTC_4 release from basophils; causes superoxide and IL-8 release from neutrophils
Eosinophil Cationic Protein (ECP)	Helminthotoxin, neurotoxin, weak RNase activity, bactericidal, virucidal; inhibits cultures of peripheral blood lymphocytes; causes histamine release from rat mast cells; neutralises heparin, alters fibrinolysis
Eosinophil –Derived Neurotoxin (EDN)	Neurotoxin, RNase activity, virucidal, weak helminthotoxin; inhibits cultures of peripheral blood lymphocytes; same as eosinophil protein X
Eosinophil Peroxidase (EPX)	In presence of H_2O_2 plus halide: kills microorganisms and tumour cells; causes histamine release and degranulation from rat mast cells; inactivates leukotrienes in absence of H_2O_2 plus halide; kills *Brugia* microfilariae; damages respiratory epithelium; provokes bronchospasm in primates. Generates reactive oxidants and free radicals

circulation persist for up to 2 weeks. They are the key mediators of immediate hypersensitivity reactions such as asthma, urticaria and anaphylaxis. In addition, they have been implicated in the delayed cutaneous hypersensitivity reaction. Basophils are stimulated by a number of mediators, such as IgE, IL-3, C5a, GM-CSF, insect venoms and morphine, to release the contents of their granules such as histamine.

The interaction between IgE and basophil/mast cell FcεIR and antigen bridging results in basophil degranulation and initiation of their effects on granule contents. The granules of basophils and mast cells contain sulfated glycosaminoglycans, responsible for their intense staining, as well as histamine, LTD_4, PAF, eosinophil chemotactic factor and kallikreins, responsible for type I immediate hypersensitivity reactions and chronic inflammation (Table 2.8). The primary protease presents in mast cells, tryptase, is mainly released during the early phase of allergic responses and is a marker of mast cell activation in chronic inflammatory diseases. Basophil and mast cell activation leads to the elaboration of cytokines such as GM-CSF, TNF-α, IFN-γ, IL-3, IL-4 and IL-5, which serve to amplify the inflammatory response.

MPS-Monocytes, macrophages, DCs and multinucleated cells

Embryonic macrophages give rise to resident tissue macrophage populations which persist in many organs after birth. Embryonic yolk sac- derived macrophages/fetal liver monocytes migrate to

Table 2.8 Basophil and mast cell granules and their contents.

Component	Function	Main physiological role	Other properties	Cell specificity
Histamine	Binds to H_1, H_2, H_3 receptors	Hypersensitivity reactions and inflammation		Basophils, mast cells
Proteoglycan Heparin	Package of basic proteins into granules	Potent anticoagulant by the activation of anti-thrombin	Binds and stabilises proteases	Connective tissue mast cells
Chondroitin sulfates	Package of basic proteins into granules	Potent mast cell inhibitor	Binds and stabilises proteases	Basophils
Enzymes Chymase	Activates precursor IL-1β	Affects microcirculation, Inflammation Modulates microcirculation Modulates skin inflammation	Apoptosis	Connective tissue mast cells
Tryptase	Activates metalloproteinase 3, inactivates fibrinogen, degrades calcitonin gene-related peptide	Proinflammatory, stimulates neutrophil chemotaxis and adherence, regulates collagenase, attenuates fibrin deposition	Tetrameric when bound to heparin, monomer inactive, restricted substrate specificity, raised levels in mast cell disorders	Mast cells
Cathepsin G-like protease Carboxypeptidase	Inactivates bradykinin	Affects microcirculation	Bactericidal	Connective tissue mast cells
Other				
Charcot–Leyden crystal protein	Lysophospholipase	Phospholipid metabolism	Neutralises pulmonary surfactant	Basophil
Major basic protein, Exoglycosidase protein Sulfatase		Disrupts membranes		Basophil

the brain (microglia), heart and skin epidermis (Langerhans cells). The brain remains relatively autonomous from bone marrow after the initial phase and microglia are maintained in adulthood by limited self-renewal. Stem cells and precursors migrate to the bone marrow in later embryonic phases and become the main source of newly recruited MPS cells in the adult, especially in the form of monocytes that mature into macrophages and DCs.

After birth, there is an initial expansion of monocytes which become the most abundant blood MPS cell, representing 2–8% of the total number of leucocytes. Monocytes are differentiated cells, but they have the ability to enter tissues and mature into macrophages. As discussed in the introduction, Monocytes originate from bone marrow pluripotent haematopoietic stem cells that commit to haematopoietic progenitor cells termed CFU-GEMM and the more committed CFU-GM). M-CSF is the most important factor in the development of monocytes although GM-CSF and IL-3 also support commitment along monocytic pathways. The CSF1R receptor, also called CD115, is a sensitive pan MPS marker, which varies in selected inflammatory states. Most CSF1R+ monocytes appear in subsets often referred to as classical and non-classical, based on CD14 and CD16 expression. CD14 is a receptor for bacterial ligands and CD16 for immunoglobulins. CSF1R+ CD14- CD16- monocytes comprise DCs in inflammation. M-CSF, IL-34 and GM-CSF are the cytokines more associated with macrophage development and GM-CSF and IL-4 for monocyte- derived DCs.

Monocytes and macrophages express a broad range of plasma membrane receptors in addition to those shared with neutrophils and described above (Figure 2.11). Particles are recognised by a combination of scavenger receptors, integrins (complement receptors), and lectins binding directly or via opsonins such as LBP, TSP, or collectins. Most of these receptors are able to recognise both pathogens and altered self-ligands such as apoptotic cells. In addition, phagocytes have specific receptors that discriminate pathogen-associated components causing inflammatory responses. For example, recognition of gram-positive bacteria is mediated by TLR2 and TLR6 and of gram-negative

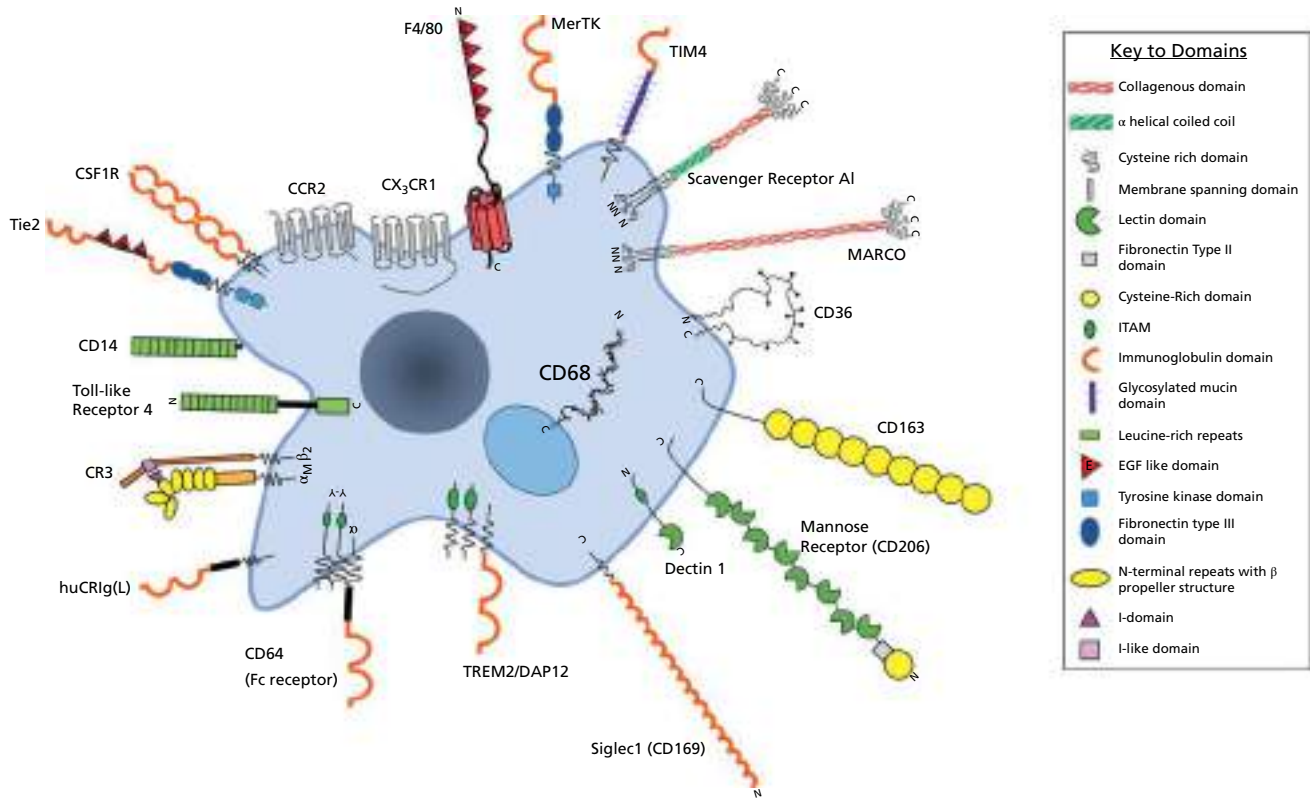

Figure 2.11 Plasma membrane antigens and receptors expressed by macrophages. Macrophages express a large number of receptors that are functionally active and can be used as markers for immunocytochemistry and FACS analysis (e.g. F4/80, CD68, CSF1 receptor, Mer-TK, CD64). The phagocytic receptors mediate elimination of microbes (e.g. MARCO), apoptotic cells (e.g. CD36, SR-A and TIM4), and circulating ligands. Other receptors bind lineage-determining cytokines, such as CSF-1. CD163 mediates the elimination of haptoglobin–haemoglobin complexes. TLR4, CD14, Dectin-1 recognise PAMPs leading to inflammation. CCR2 and CX3CR1 are chemokine receptors highly expressed in macrophages. Siglec-1 (CD169), a receptor for sialic acid terminal glycoconjugates, mediates adhesion of host cells and microbes, whereas CD206, a receptor for clearance of mannosyl terminal glycoproteins, is a prototypical marker of M2-type activation. The scavenger receptor SR-A binds polyanionic ligands such as oxidised LDL, as well as microbes. CD36 mediates adhesion and giant cell formation. For further details, see BMC, with permission (Source: Gordon *et al.* 2020/John Wiley & Sons.)

bacteria by TLR4. In contrast, anti-inflammatory signals are triggered after binding of apoptotic cells that expose phosphatidyl-serine (PS) on their cell surface. Ligation of receptors for apoptotic cells (especially the receptor for PS) causes liberation of TGFβ and other immunomodulatory cytokines. Hence, multiple receptors are involved in recognition with certain common receptors mediating internalisation of both pathogens and self-ligands. Together these receptor complexes contribute to discriminating and initiating appropriate responses.

Activation of macrophages with IFN-γ, produced by NK and Th1 cells results in a microbicidal 'classical' macrophage activation state, whilst IL-4 and IL-13 produced by the Th2 population of T lymphocytes induce an 'alternative' form of macrophage activation, promoting antigen-presentation (Figure 2.12). These cytokines then enhance macrophage stimulation of T and B lymphocytes by inducing class II MHC antigen and costimulatory molecule expression. Activated macrophages, in turn, produce cytokines that stimulate both types of helper T cells. Several cytokines, including IL-4, IL-10, IL-13 and TGF-β, can inhibit different aspects of macrophage function. Furthermore, prostaglandin (PG)E_2, elaborated by macrophages, as well as corticosteroids can suppress various actions of macrophages. These provide a feedback mechanism for control of the immune response.

Dendritic cells (DCs) were first described as rare, distinct mononuclear cells in mouse spleen by Steinman and Cohn in 1973. The new cell type was characterised by large size, relatively smooth surface and a convoluted nucleus containing small nucleoli. In addition, these motile cells presented a cytoplasm rich in large mitochondria and vesicles arranged in multiple plasma membrane processes of various extensions that

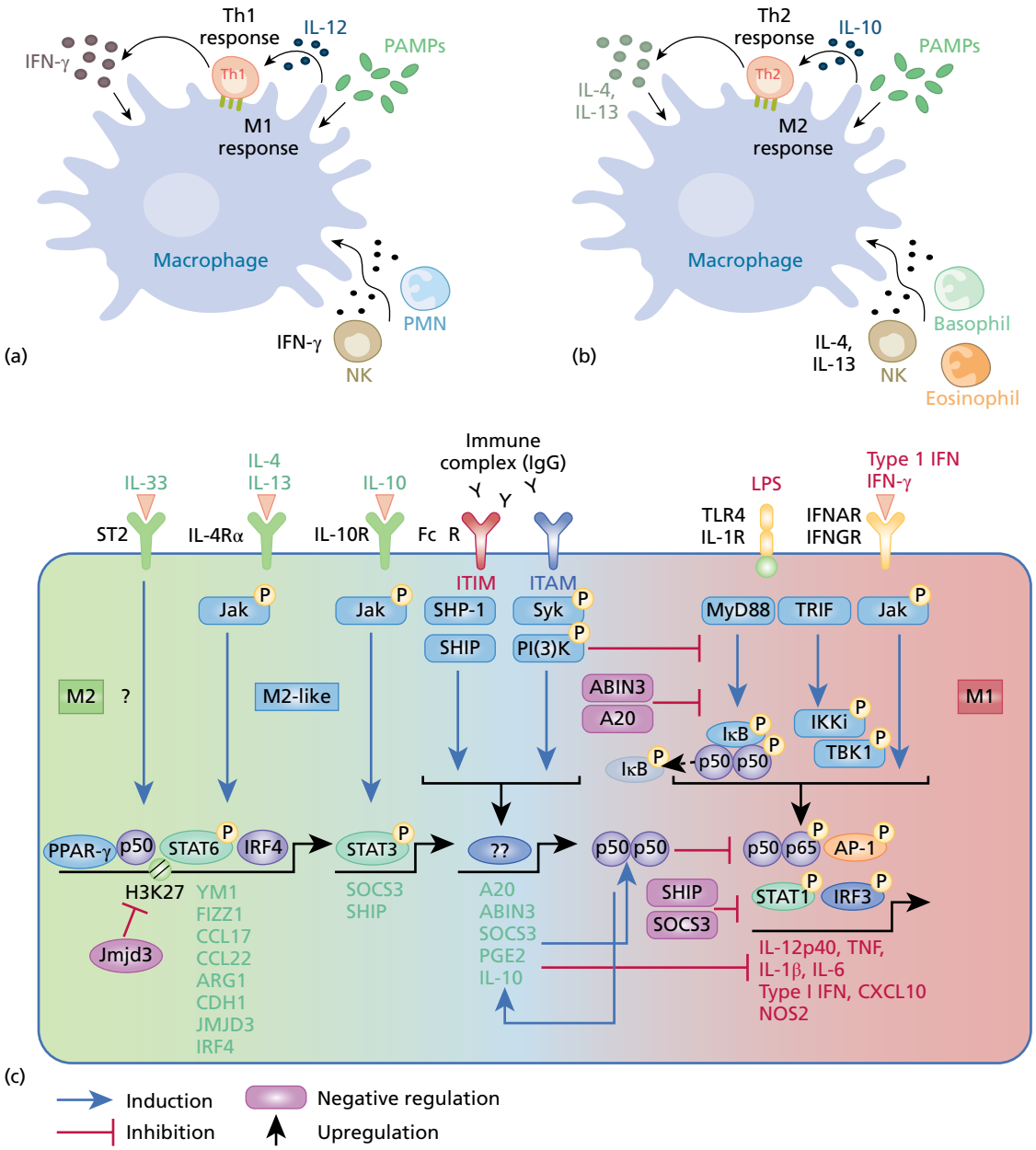

Figure 2.12 Two-signal macrophage activation model in the context of the immune response. (a) Macrophage full activation requires two major signals, recognition of PAMPs by PRRs and stimulation with IFN-γ or IL-4, in a cycle that determines the development of specificity and effector functions in the immune response. For Th2 responses. (b), uncharacterised PRRs induce the recruitment of IL-4 and IL-13, producing innate immune cells such as eosinophils and basophils and naive T lymphocytes. IL-4 and IL-13 produced by innate immune cells induce a first wave of innate alternative activation in macrophages but also provide the secondary signal for Th2 development. (c) The major pathways of macrophage polarisation are outlined (Source: Adapted from Martinez *et al.* 2009; Sica and Mantovani 2012.)

they termed 'dendrites'. Unlike macrophages, DCs lacked abundant membrane ATPase, digestive bodies and lysosomes, and displayed less FcR-dependent phagocytic activity compared with macrophages, both *in vivo* and *in vitro*. Although far less numerous than tissue macrophages, DCs expressed a potent and unique ability to induce primary immune responses to foreign antigens in naive lymphocytes, whereas both DCs and macrophages can activate primed lymphocytes by capture, processing and presentation of MHC-bound peptides to T and B cell antigen-specific receptors. DCs have a rapid turnover in

the spleen, and are only transiently adherent to culture vessels, compared with macrophages, and are difficult to maintain in standard media in vitro.

Tissue-based DC performs predominantly antigen-presenting rather than phagocytic clearance functions. Langerhans cells are found in virtually all tissues except the brain and are the major immunological cellular components of the skin and mucosa. Their racquet-shaped ultrastructural inclusions (Birbeck bodies) distinguish them from other tissue cells. They interact with and process antigen, then migrate to lymphoid organs where, through interaction with T cells, they generate cellular and humoral immune responses. This ability of DCs to interact with T cells and other inflammatory cells contributes to the often-varied clinical manifestations of the histiocytic disorders.

The presence and functions of DCs have been described in many organs and animal species, as well as in human blood. Further studies revealed that human DC constitutively expresses high levels of class I and II major histocompatibility complex (MHC) molecules and many other markers, such as CD58, CD54, CD50, CD40, CD80 and CD86. CD58, CD54, CD50 and CD40 are adhesion molecules essential for the binding with T cells; they bind to CD2, CD11a, integrin LFA-1 and CD154, respectively. CD80 and CD86 are costimulatory molecules for T cell activation; they both bind to CD28. DCs were soon found to be in the T-cell areas of peripheral lymphoid organs such as the spleen, lymph nodes and Peyer's Patches, the ideal location for initiating immune responses.

Disorders of the phagocyte system

Neutrophil congenital disorders (Table 2.9)

Primary neutropenic disorders are rare and heterogeneous conditions presenting as *Severe Congenital Neutropenia* (SCN) or as *Cyclic Neutropenia* (CyN), a milder disease. A unifying feature of these conditions is premature apoptosis of neutrophil progenitors and precursors, leading to differentiation/maturation blockade. Half of SCN cases are linked to defects in the gene encoding neutrophil elastase (*ELANE*), one of the four major serine proteases synthesised in neutrophil granule formation. Various SCN forms can predispose to the myelodysplastic syndrome (MDS) and acute myelogenous leukaemia (AML) by mechanisms involving acquired, activating mutations in the G-CSF receptor gene and in other leukaemia-associated genes. SCN is usually diagnosed in infancy in the setting of recurrent severe infections. First-line treatment of infections in such patients is antibiotics with G-CSF. Stem cell transplantation offers the potential to cure for those who do not respond to antibiotics and G-CSF.

Multisystemic disorders with neutropenia comprise conditions in which neutropenia is only one of the consequences of alterations in general cell functions, such as primary mitochondrial dysfunctions (Table 2.9). Examples are the *Shwachman–Diamond–Oski syndrome* and *Charcot-Marie-Tooth disease*, a relatively common inherited disorder of the peripheral nervous system. WHIM (warts, hypogammaglobulinaemia, infection, myelokathesis) is characterised by increased retention (kathésis) of myeloid cells in the bone marrow due to increased sensitivity of the CXCR4 receptor to CXCL12.

Neutrophil dysfunction disorders involve defects in specific neutrophil functions such as adherence to the vascular endothelium (*Leucocyte Adhesion Deficiency* or LAD) and generation of reactive oxygen species (*Chronic Granulomatous Disease* or CGD). LAD is characterised by defects in adhesion of neutrophils to blood vessel walls with decreased migration from the bloodstream to sites of infection despite persistent leucocytosis, delayed separation of the umbilical cord and impaired wound healing. CGD is characterised by defects in the phagosomal and plasma membrane-associated NADPH oxidase, resulting in impaired superoxide formation, decreased killing of catalase-positive organisms and chronic infection.

Myeloperoxidase (MPO) deficiency, the most common inherited disorder of phagocytes, is associated with a higher incidence of fungal infections.

Secretory granule and lysosome disorders involve defects in endosomal/lysosomal maturation, trafficking and content. *Chédiak–Higashi syndrome* is a rare autosomal recessive disorder characterised by oculocutaneous albinism, recurrent and severe bacterial infections and giant blue–grey granules in the cytoplasm of white blood cells (Figure 2.13).

Autoinflammatory disorders are caused by an increased activation of the innate immune system, leading to episodes of fever and localised or systemic inflammation in the absence of infection or other inflammatory stimuli (see Table 2.10). These conditions should be distinguished from autoimmune diseases, which are caused by increased activation of the adaptative (as opposed to innate) immune system with the production of antibodies or T cells reacting against autologous or self-antigens.

Disorders of monocyte/macrophages: a genetic perspective

The recruitment of bone marrow-derived monocytes by sterile, infectious and immune stimuli gives rise to inflammation, with local and systemic impacts on tissue perfusion, thermoregulation and hepatic biosynthesis, innate and acquired immune activation, tissue injury and repair (Nathan 2022). Macrophages are extensively activated by pro-inflammatory cytokines such as IL-1β, IL6, TNF alpha and type 1 interferons, as well as anti-inflammatory IL-10, TGF beta cytokines and arachidonate metabolites. Widespread vaccination with live BCG brought to light the importance of macrophage activation by gamma interferon, IL-12 and IL-18 cytokines in

Table 2.9 Inherited neutrophil disorders.

Gene protein	Disease name	Inheritance pattern	Molecular mechanisms
Disorders with primary neutropenia			
ELANE neutrophil elastase	Congenital neutropenia or cyclic neutropenia	AD or somatic	Maturation arrest, premature apoptosis unfolded protein response, ER stress
JAGN1 Jagunal homolog 1	Congenital neutropenia	AR	Differentiation defect, premature apoptosis ER secretory pathway
CSF3R colony-stimulating factor receptor	Congenital neutropenia	AR and AD	Bone marrow production and release signalling defect
GFI1 growth factor independence 1	Congenital neutropenia	AD	Myeloid cell differentiation Transcription repressor
G6PC3 Glucose-6-phosphatase	Congenital neutropenia	AR	Bone marrow retention, apoptosis ER stress, glycosylation defect
HAX1 HS1-associated protein X1	Kostmann syndrome	AR	Bone marrow production and release
Multisystemic syndromes with neutropenia			
AK2 Adenylate kinase 2	Reticular dysgenesis	AR	Differentiation defect Mitochondrial metabolism dysfunction
RMRP RNAase mitochondrial RNA processing	Cartilage hair hypoplasia	AR	Bone marrow dysfunction Preribosomal RNA processing
SBDS Shwachman–Bodian–Diamond syndrome protein	Shwachman–Bodian–Diamond syndrome	AR	Differentiation defect, premature apoptosis Ribosome biogenesis
DNM2 Dynamin 2	Charcot-Marie-Tooth disease	AD	Membrane trafficking, microtubules
TAZ1 Tafazzin	Barth syndrome	XLR	Mitochondrial membrane dynamics
G6PT Glucose-6-phosphate transporter	Glycogen storage disease type 1b	AR	Premature apoptosis ER stress, mitochondrial dysfunction
Immunodeficiency syndromes with neutropenia			
BTK Bruton's tyrosine kinase	X-linked agammaglobulinaemia	XLR	Chemotaxis defect, reactive oxygen defect
WAS Wiskott-Aldrich syndrome	X-linked neutropenia Wiskott-Aldrich syndrome	XLR	Decreased proliferation, increased apoptosis Actin polymerisation
CD40L CD40 ligand	Hyper-IgM syndrome	XLR	Adhesion and transmigration
CXCR4 Chemokine receptor CXCR4	WHIM (warts, hypogammaglobulinaemia, immunodeficiency, and myelokathexis)	AD	Bone marrow and tissue homing abnormality Defective chemokine receptor function
STK4 Serine/threonine kinase 4	STK4 deficiency	AR	Increased apoptosis Mitochondrial dysfunction
GINS1 Go-ichi-ni-san complex subunit 1	GINS1 deficiency	AR	Impaired cell cycle Defective DNA repair

(*Continued*)

Table 2.9 (*Continued*)

Gene protein	Disease name	Inheritance pattern	Molecular mechanisms
Neutrophil dysfunction disorders			
ITGB2 Leucocyte integrin β2 chain	LAD-I	AR	Neutrophil adhesion/migration defects Adhesion molecule deficiency
GFTP *GDP* fucose transporter	LAD-II	AR	Selectin deficiency Defect in fucose transport
FERMT3 *KINDLIN*-3	LAD-III	AR	Neutrophil adhesion defect
CYBB Cytochrome b-245β; gp91phox	Chronic granulomatous disease	XLR	Defective oxidative burst NADPH oxidase enzyme defect
CYBA Cytochrome b-245α; p22phox	Chronic granulomatous disease	AR	Defective oxidative burst NADPH oxidase enzyme defect
NCF1 Neutrophil cytosolic factor-1; p47phox	Chronic granulomatous disease	AR	Defective oxidative burst NADPH oxidase enzyme defect
NCF2 Neutrophil cytosolic factor-2; p67phox	Chronic granulomatous disease	AR	Defective oxidative burst NADPH oxidase enzyme defect
NCF4 Neutrophil cytosolic factor-4; p40phox	Chronic granulomatous disease	AR	Defective oxidative burst NADPH oxidase enzyme defect
G6PD Glucose-6-phosphate dehydrogenase	G6PD deficiency	AR	Defective oxidative burst, NETosis defect Enzyme deficiency
RAC2 Ras-related C3 botulinum toxin 3	Neutrophil immunodeficiency syndrome	AR	GTPase deficiency, defective oxidative burst Secretory and phagocytosis defect
MYD88 Myeloid differentiation 88	MyD88 deficiency	AR	Neutrophil aging TLR and IL-1R signalling defect
IRAK4 Interleukin-1 receptor-associated kinase 4	IRAK4 deficiency	AR	Defective migration and phagocytosis Impaired TLR and IL-1 receptor responses
Secretory lysosome/granule defects			
LYST Lysosomal trafficking regulator	Chediak-Higashi syndrome	AR	Neutrophil signalling defect Abnormal lysosome and melanosome trafficking
RAB27A *RAB27a*	Griscelli syndrome type 2	AR	Reduced mature neutrophils Membrane trafficking/phagosome secretion defect
UNC13D *MUNC13*-4	Familial haemophagocytic lymphohistiocytosis type 3	AR	Neutropenia, vesicular trafficking, and secretion defects
STXBP2 Syntaxin binding protein 2 (MUNC18-2)	Familial haemophagocytic lymphohistiocytosis type 5	AR	Secretion and bactericidal defects
WDR1 Actin-interacting protein 1 (Aip1)	WDR1 deficiency	AR	Mild neutropenia, impaired chemotaxis Normal bacterial killing and increased oxidative burst
MLK1 Megakaryoblastic leukaemia 1 (MKL1)	MLK1 deficiency	AR	Decreased phagocytosis and impaired migration

AP3B1 Adaptor-related protein complex β1	Hermansky-Pudlak syndrome	AR	Reduced mature neutrophils Abnormal vesicular trafficking of proteins
LAMTOR2 Late endosomal/lysosomal adaptor, MAPK and MTOR activator 2	Immunodeficiency due to defect in MAPBP-interacting protein (p14 deficiency)	AR	Abnormal neutrophil maturation and function Late endosome biogenesis
CEBPE CCAAT enhancer binding protein ε	Specific granule deficiency	AR	Neutrophil chemotaxis defect Abnormal or absent granule formation
Autoinflammatory disorders			
NLPR3 Cryopyrin	Cryopyrin-associated periodic syndromes (CAPS)	AD or somatic	Neutrophil homeostasis dysregulation Inflammasome mediated IL-1β release/cell death
MEFV Pyrin	Familial Mediterranean fever	AR or rarely AD	Neutrophil chemotaxis and phagocytosis defect Enhanced apoptosis and altered adhesion Hyperactive inflammasome-mediated IL-1β release Alterations in F-actin dynamics
MVK Mevalonate kinase	Mevalonic aciduria and hyper-IgD syndrome	AR	Pyrin inflammasome activation due to RhoA inactivation and compromised phosphatidylinositol 3-kinase activity secondary to prenylation defect
TNFRSF1A Tumour necrosis factor receptor–1A	Tumour necrosis factor receptor–associated periodic syndrome	AD	Neutrophil apoptosis resistance TNFR signalling or shedding defect
NOD2 Nucleotide oligomerisation domain 2	Blau syndrome	AD	Increased ocular neutrophil rolling and adherence Activation of nuclear factor κB/IL-1β –mediated inflammation
IL1RN Interleukin-1 receptor antagonist	Deficiency of IL-1 receptor antagonist	AR	Neutrophil mobilisation and activation Unregulated IL-1 receptor activation
CD2BP1 CD2 binding protein 1	Pyogenic arthritis pyoderma gangrenosum and acne (PAPA)	AD	Neutrophil mobilisation and activation Defective actin dynamics
PSMB8 Proteasome subunit β type 8	Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE)	AR	Neutrophil mobilisation and activation IFN dysregulation
TMEM173 Stimulator of interferon genes (STING)	STING-associated vasculopathy with onset in infancy (SAVI)	AD	Neutrophil mobilisation and activation IFN dysregulation
Other disorders			
DNASE1 Deoxyribonuclease 1	Monogenic systemic lupus erythematosus	AR	Defective NET degradation Increased ROS production IFN dysregulation
DNASE1L3 Deoxyribonuclease 1L3	Monogenic systemic lupus erythematosus	AD	Defective NET degradation Increased ROS production IFN dysregulation
CFTR Cystic fibrosis transmembrane regulator	Cystic fibrosis	AR	Delayed neutrophil apoptosis Impaired MPO activity

(*Source:* Ley *et al*. 2018/American Association for the Advancement of Science.)

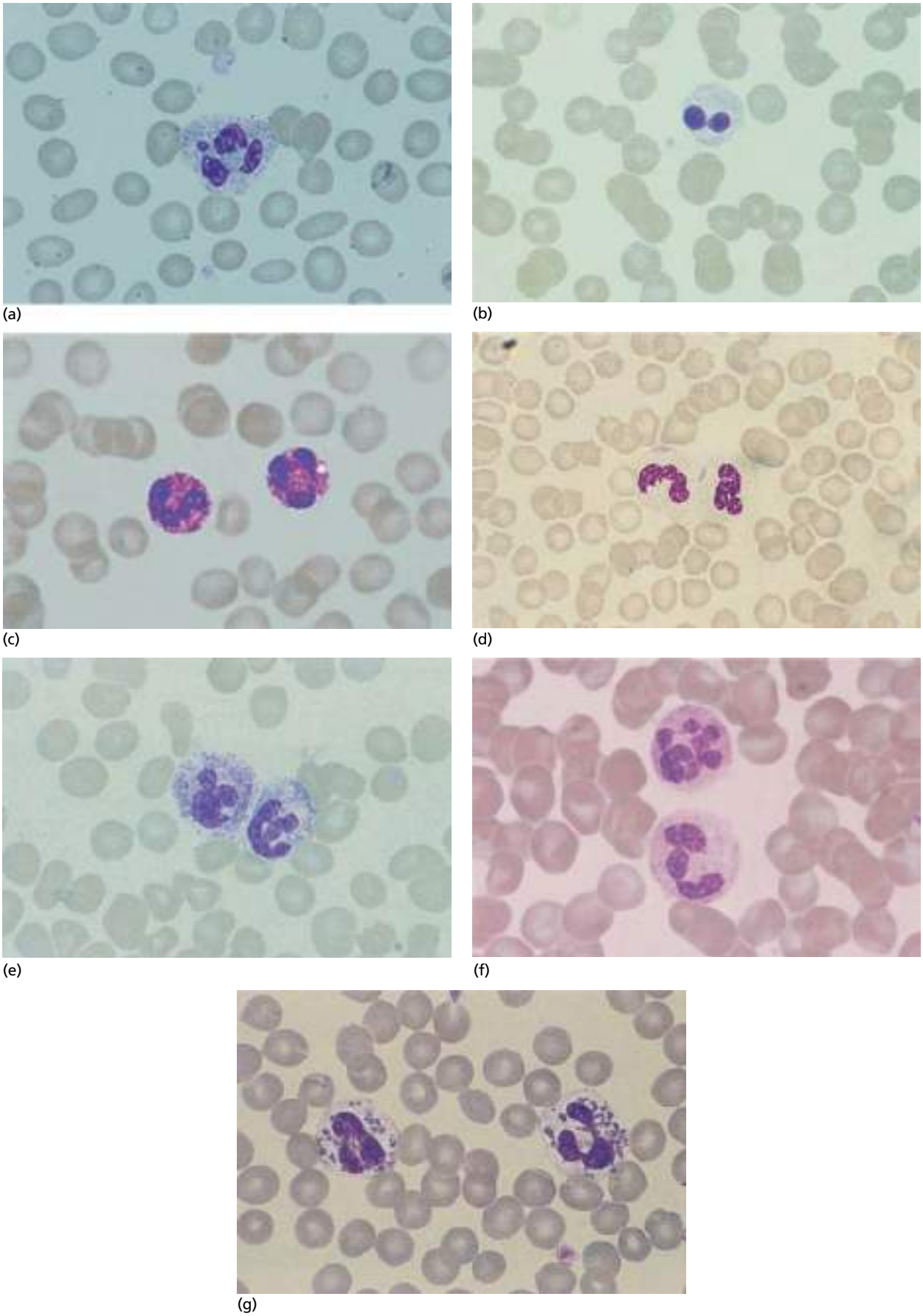

Figure 2.13 Variations in neutrophil morphology. Some nuclear morphologic changes may result from abnormalities in packaging of chromosomal centromeric DNA within a single nucleus (a) Barr body, a drumstick appendage to the nucleus. (b) Pelger–Huët anomaly with a bilobed nucleus. (c) Alder–Reilly anomaly with prominent purple granules (also in monocytes and lymphocytes). (d) May–Hegglin anomaly with Döhle bodies in the cytoplasm. (e) Toxic granulation. (f) Neutrophil with nuclear hypersegmentation and a normal neutrophil. (g) Chédiak–Higashi syndrome with giant granules. https://imagebank.hematology.org/collection/3214.

genetic resistance mediated by cellular adaptive immunity to infection. The discovery of inflammasome assembly and activation of IL-1 processing in macrophages by Tschopp and Martinon Figure 2.5 and (Martinon *et al*. 2002),) catalysed subsequent elucidation of the mechanisms that underlie a growing number of innate auto-inflammatory genetic syndromes (Table 2.10). This occurred in parallel with the link observed between type1 interferons, auto-immune diseases and antiviral resistance, especially in severe COVID-19 susceptibility (Zhang *et al*. 2022).

Table 2.10 lists monogenic inflammatory syndromes associated with inflammasome and other pro-inflammatory cytokine receptor and signalling disorders, cytoskeletal dysregulation and phospholipase C gamma2 function. Further details of age of onset and clinical manifestations are given in (Savic *et al*. 2022). Table 2.11 lists monogenic Type 1 interferonopathies (Savic *et al*. 2022; Zhang *et al*. 2022) and Figure 2.14 categorises putative functions of genes involved in this cellular pathway (Crow and Stetson 2021). IL-1 R antagonist and a range of anti-cytokine/R antibodies provide important therapeutic options, supplemented by corticosteroids and bone marrow transplantation where indicated. Type1 interferon genetic deficiency has been found in about 2% of subjects infected by COVID-19, exacerbating the risk of severe illness and death; anti-interferon antibodies arise during infection in about 10% of individuals without a genetic deficiency in the type1 interferon pathway, mimicking the effect of its absence (Zhang *et al*. 2022).

Table 2.10 Monogenic autoinflammatory syndromes.

General pathway	Disease	Gene	Affected protein/ function	Mode of inheritance	Treatment
			Inflammasomopathies		
Pyrinopathies	FMF	*MEFV*	Pyrin	AR/AD	Colchicine/anti-IL1
	PAAND	*MEFV*	Pyrin	AD	Anti-IL-1/anti-TNF
	MKD	*MVK*	Mevalonate kinase	AR	NSAIDs/prednisolone/ anti-IL-1/ anti-TNF
	PAPA	*PSTPIP1*	PSTPIP-1/CD2 binding protein-1	AD	Prednisone/anti-IL-1, anti-TNF
NLRP3-AID (CAPS)	Mild (FCAS1)	*NLRP3*	NLRP3/NALP3	AD	Anti-IL-1
	Moderate (MWS)	*NLRP3*	NLRP3/NALP3	AD	Anti-IL-1
	Severe (NOMID/CINCA)	*NLRP3*	NLRP3/NALP3	AD	Anti-IL-1
	NLRP12-AID (FCAS2)	*NLRP12*	Monarch-1 protein	AD	IL-1 blockade/corticosteroids
	NLRC4-AID	*NLRC4*	NLRC4	AD	Anti-IL-1/anti-IL18
	NLRP1-AID	*NLRP1*	NLRP1	AD	Acitretin, anti-IL-1
Disease	**Gene**	**Affected protein/ function**	**Mode of inheritance**	**Treatment**	
		Others			
TRAPS – high penetrance	*TNFRSF1A*	TNF receptor	AD	Corticosteroids/ anti–IL-1/ anti–IL-6/etanercept	
TRAPS – low penetrance	*TNFRSF1A*	TNF receptor	AD	Anti-TNF	
DIRA	*IL-1RN*	IL-1 receptor antagonist	AR	Anti-IL-1	
DITRA	*IL-36RN*	IL-36 receptor antagonist	AR	anti–IL-1, anti-TNF, anti–IL-12/23, anti–IL-17	
NOD2-AID	*NOD2*	NOD-like receptor protein	AD, SM	methotrexate, anti-TNF, anti–IL-1, anti–IL-6, corticosteroids	
Monogenic Systematic Still's	*LACC1* *IL1RN*			IL-6 blockade, IL-1 blockade, glucocorticoids	
FACAS	*F12*	Human coagulation factor XII	AD	IL-1 blockade bradykinin-B2 blockade	

(*Continued*)

Table 2.10 (*Continued*)

Disease	Gene	Affected protein/ function	Mode of inheritance	Treatment
		Relopathies		
Haploinsufficiency of A20 (HA20)	*TNFAIP3*	NF-kB regulatory protein, A20	AD (some cases unclear)	Colchicine, systemic corticosteroids, anti–IL-1, anti– IL-6, anti-TNF, baricitinib
ORAS/otulipenia	*OTULIN*	Otulin; (de-uniquitinator protease)	AR	Anti-TNF, corticosteroids, anti–IL-1
RELA (p65) haploinsufficiency	*RELA*	relA (p65) NF-kB subunit	AD	Anti-TNF
NEMO partial deficiency	*IKBKG*	NF-kB essential modulator	XL	IgG replacement, antibiotics, HSCT, anti-TNF
Biallelic RIPK1 mutations	*RIPK1*	Receptor-interacting serine/threonine kinase 1	AR	HSCT
HOIL-1/HOIP deficiency	*HOIL1, HOIP*	HOIP, HOIL-1, SHARPIN (components of LUBAC)	AR	IgG replacement, antibiotics, anti-TNF, anti–IL-1, corticosteroids
NFKB1-AID	*NFKB1*	P50/p105 NF-kB subunit	AD	N/A

Disease	Gene	Affected protein/ function	Mode of inheritance	Treatment
		Actin cytoskeleton dysregulation		
Autoinflammatory periodic fevers, immunodeficiency and thrombocytopenia (PFIT)	*WDR1*	WD repeat domain 1; degradation of intermediate filaments, polymerisation of F-actin	AR	Colchicine, corticosteroids, anti–IL-1, HSCT
ARPC1B deficiency	*ARPC1B*	Actin-related protein 2/3 complex subunit 1B; branching of F-actin	AR	Antibiotics corticosteroids, mycophenolate, sirolimus, HSCT

Disease	Gene	Affected protein/ function	Mode of inheritance	Treatment
		PLCG2 AID		
PLAID	*PLCG2*	Phospholipase Cy2	AD	Symptomatic (e.g. IVIg, cold avoidance)
APLAID	*PLCG2*	Phospholipase Cy2	AD	Corticosteroids, anti–IL-1
DADA2	*CECR1*	Adenosine deaminase 2	AR	Anti-TNF, bone marrow transplantation

Disease	Gene	Affected protein/ function	Treatment
		Adult onset autoinflammatory diseases	
VEXAS syndrome	*UBA1*	Met41 ubiquitylation	Glucocorticoids, IL-6 blockade, bone marrow transplantation
ASOD	Unknown	Unknown	IL-6 blockade, IL-1 blockade, glucocorticoids
Schnitzler syndrome	Unknown	Unknown	IL-1 blockade

AD, autosomal dominant; AID, associated inflammatory disease; AOSD, adult-onset still's disease; AR, autosomal recessive; CAPS, cryopyrin-associated periodic fever syndromes; DADA2, deficiency of adenosine deaminase 2; DIRA, deficiency of IL-1 receptor antagonist; DITRA, deficiency of the IL-36 receptor antagonist; FACAS, factor XII–associated cold autoinflammatory syndrome; FCAS2, familial cold autoinflammatory syndrome 2; FMF, familial Mediterranean fever; HSCT, haematopoietic stem cell transplantation; LUBAC, linear ubiquitin chain assembly complex; MKD, mevalonate kinase deficiency; N/A, not available; NSAID, nonsteroidal anti-inflammatory drug; ORAS, otulin-related autoinflammatory syndrome; PAAND, pyrin-associated autoinflammation with neutrophilic dermatosis; PAPA, pyogenic arthritis, pyoderma gangrenosum, and acne syndrome; TRAPS, TNF-associated periodic fever syndrome; VEXAS, vacuoles, E1 enzyme, X-linked, autoinflammation, somatic.
(*Source:* Adapted from Savic *et al.* 2022).

Table 2.11 Monogenetic interferonopathies.

Disease	Gene	Affected protein/ function	Mode of inheritance	Treatment
		Interferonopathies		
CANDLE/PRAAS	*PSMA3*, *PSMB4* *PSMB8-9*, *POMP*	Proteasome	AR	Glucocorticoids, Jak inhibition
Aicardi Goutieres syndrome (AGS)	*TREX1*, *RNASEH2A-2C*, *SAMHD1ADAR1IFIH1*	Exonuclease subunits of the RNase H2 endonuclease complex Control of dNTP pool	AR/AD	Symptomatic treatment, Jak inhibition, reverse transcriptase inhibitors
SAVI	*TMEM173*	Stimulator of Interferon Genes (STING)	AD, SM	Jak inhibition
DNASE2 deficiency	*DNASE2*	Deoxyribonuclease	AR	Jak inhibition

AD, autosomal dominant; AR, autosomal recessive; CANDLE, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome; DADA2, deficiency of adenosine deaminase 2; SAVI, sting-associated vasculopathy with onset in infancy (Savic *et al.* 2022).

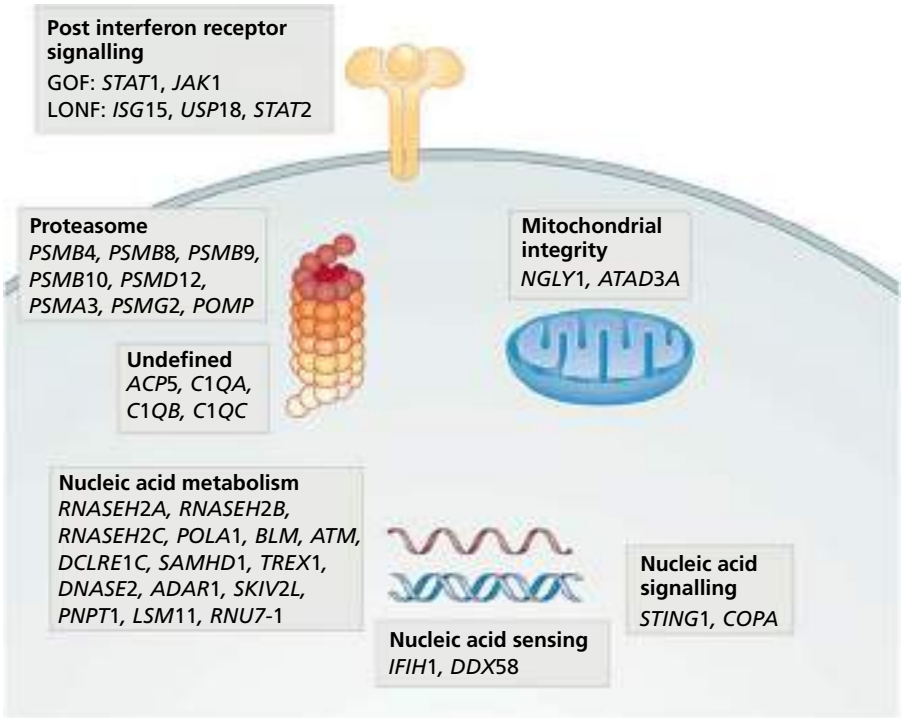

Figure 2.14 Type I interferonopathy genotypes according to the function of the encoded gene products. Putative type I interferonopathy mutated gene products act directly on nucleic acid substrates (nucleic acid 'metabolism') or are involved in nucleic acid sensing; others have a primary role in transducing a DNA signal to an innate immune response, are involved in downstream regulation of type I interferon receptor signalling or contribute to the maintenance of mitochondrial integrity. Further genes relate to the proteasome, where the precise link to type I interferon induction remains unclear. The relationship of mutations in *ACP5* and *C1QA*, *C1QB*, and *C1QC* mutations to disease remains unproven. GOF, gain of function; LONF, loss of negative feedback. Further details are given in (Source: Crow and Stetson 2021/Springer Nature).

Conclusion

Phagocytes provide a unique and integral contribution to all aspects of haematologic research and clinical practice. Ready access to blood has brought new insights into the origin, differentiation and activation of the distinct, but related phagocyte lineages. Hitherto, haematologists have focussed mainly on cells in blood and bone marrow, yet all mature leucocytes have a significant presence and functional role within lymphohaematopoietic and other organs, contributing to homeostasis as well as pathology. Tissue phagocytes must not be overlooked. Many of the rare monogenic genetic disorders listed above become manifest in early life, obscuring the contribution of genetics to diseases associated with later life. Common pathologies such as atherosclerosis, malignancy and neurodegenerative disease develop gradually over many years and mononuclear phagocytes contribute substantially to their pathogenesis in blood and tissues.

Acknowledgements

The authors thank Annabell Roberti for help with preparing the manuscript.

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CHAPTER 3

Erythropoiesis in the human

3

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Introduction

Erythropoiesis is the production of red blood cells carrying the respiratory pigment haemoglobin for the transport of oxygen to tissues. This process involves the commitment of multipotent haematopoietic stem cells (HSCs) to the erythroid lineage followed by expansion and differentiation of committed erythroid cells. The early stages of erythropoiesis are governed by complex transcriptional and epigenetic programmes which respond to extracellular and intracellular signalling. In the later phases of erythropoiesis, multiple events occur including the synthesis of haemoglobin, condensation and extrusion of the nucleus, and the elimination of most organelles to produce reticulocytes. Reticulocytes then undergo membrane and cytoskeletal remodelling and removal of any remaining organelles before forming mature erythrocytes. This entire process normally maintains the steady state of an individual's red cell mass, producing $1–2 \times 10^{12}$ new red cells (25–50 mL of packed red cells) per day to replace those that are lost through senescence or premature destruction. Any failure to maintain the balance between production and loss results in anaemia which is one of the most common manifestations of a very wide range of inherited and acquired disorders affecting up to a third of the world's population at some time and commonly for a long period of their lives.

Understanding the basic biology of erythropoiesis provides a logical framework for the diagnosis and treatment of inherited and acquired anaemias. Many of the principles underlying normal erythropoiesis have been established by studying model organisms particularly mouse, chicken and zebrafish. Important information has also been obtained by studying cell lines, from a variety of species, which can expand and differentiate along the erythroid pathway. However, the details of erythropoiesis in these experimental models often differ between species, and cell lines do not always reflect the mechanisms that underly normal erythropoiesis *in vivo*. In addition, cell models usually recapitulate red cell production at specific developmental stages (embryonic, fetal or adult) at which times certain aspects of erythropoiesis may be regulated quite differently. Over the last 20 years, our understanding of erythropoiesis in humans has been greatly improved via human genetic studies. Genes and pathways mutated in individuals with erythroid disorders can now be rapidly identified by DNA sequencing. Furthermore, genome-wide association studies have identified many new modifiers of erythropoiesis. In addition, we can now expand primary erythroid cells from progenitors obtained from peripheral blood and this has enabled detailed molecular and cellular analysis of both normal and disordered erythropoiesis in humans. Such developments are not only increasing our understanding of normal erythropoiesis, but also improving our ability to diagnose and treat red cell disorders. In this chapter, we outline the normal mechanisms underlying erythroid specification, differentiation and maturation in human erythropoiesis and highlight some points at which this complex system may fail.

The developmental origins of erythropoiesis

Based on analysis of both human and mouse, erythropoiesis has been shown to occur in three successive waves during development. Each wave emerges from a different site in the developing embryo, and the first wave begins at the same time

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).

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as development of the circulatory system. Primitive erythropoiesis (PE) is first evident in humans at around 3 weeks post conception and arises from the blood islands of the extra-embryonic yolk sac. By analogy to the mouse, a second wave of haemopoietic activity is thought to emerge from erythromyeloid progenitor (EMP) cells in the yolk sac at approximately 4 weeks of gestation and this marks the onset of definitive erythropoiesis (DE). Erythroid progenitors released into the circulation at this time pass to the liver, which becomes the main site of definitive erythropoiesis in the fetus. A final wave of definitive haemopoietic activity occurs in the aorta-gonad–mesonephros (AGM) region, the placenta and the major blood vessels at approximately 4–6 weeks of gestation. By 10–12 weeks, haemopoiesis starts to migrate to the bone marrow, where blood formation becomes fully established during the last 3 months of gestation (see Chapter 1 for further details).

Primitive and definitive erythroid cells are distinguished by their morphology, cytokine responsiveness, growth kinetics, transcription factor programmes, epigenetic programmes, regulation of cell cycle and patterns of gene expression. Definitive fetal erythroid cells also differ from adult erythroid cells. Importantly, the types of haemoglobin produced are quite distinct in human embryonic (Hb Gower I $\zeta_2\varepsilon_2$, Gower II $\alpha_2\varepsilon_2$ and Hb Portland $\zeta_2\gamma_2$), fetal (HbF $\alpha_2\gamma_2$) and adult (HbA $\alpha_2\beta_2$ and HbA2 $\alpha_2\delta_2$) erythroid cells (Figure 3.1). These specific patterns of globin expression provide key markers for identifying the developmental stages of erythropoiesis: in particular, embryonic haemoglobins are not produced at all in normal definitive cells (EMP and DE). It remains unclear whether primitive and definitive haemopoiesis in mammals have entirely separate origins or if they are both derived from common stem cells that arise during very

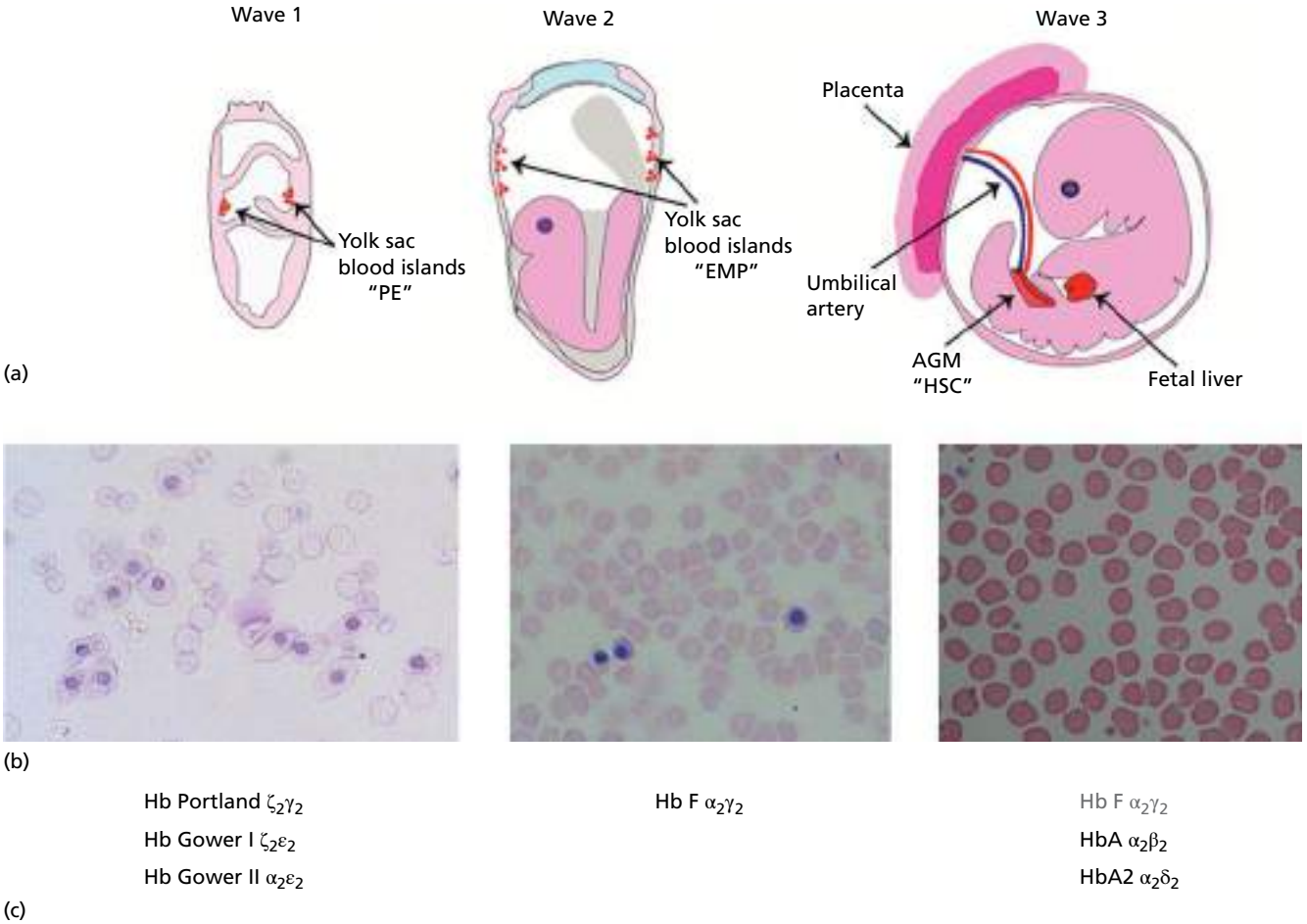

Figure 3.1 The three waves of primitive (PE), erythromyeloid (EMP) and definitive erythropoiesis. (a) shows the origins of erythropoiesis during PE, EMP and DE. (b) shows the appearance of human peripheral blood at 8 and 14 weeks post conception and in the adult. At 14 weeks some nucleated embryonic cells are still found in the circulation amidst polychromatic red cells of fetal DE. (c) illustrates the major types of haemoglobin seen at each stage of development.

early development. The close proximity of the blood and endothelial cells in the yolk sac suggests that haematopoietic cells and blood vessels originate from a common precursor. Accurately defining the embryological origins of these lineages (see Chapter 1) will be important for understanding the normal mechanisms that establish and maintain HSCs and how these programmes are subverted in common haematological disorders.

The cellular and molecular pathways leading to definitive erythropoiesis

Multipotent HSCs represent about 1 in 10,000 nucleated cells in the bone marrow. The precise mechanism by which definitive, AGM-derived HSCs undergo lineage commitment to multipotential and bi-potential populations of progenitors in the bone marrow is still under investigation and several models are currently proposed (see Chapter 1). The original concept of a strict hierarchical, stepwise progression of lineage specification and differentiation has been replaced over recent years by more nuanced and diverse models. For example, it has been suggested that commitment may be a continuous rather than a stepwise process and that some HSCs are primed to progress immediately to form erythroid cells, megakaryocytes and platelets rather than pass through the more extensive network of intermediate stages. However, at least some HSCs differentiate by passing through early stages as multipotential progenitors such as colony forming units (CFUs) that have the ability to produce granulocytes (G), erythrocytes (E), monocytes (M) and megakaryocytes (Meg) (CFU-GEMM). Further differentiation progressively restricts the lineage potential of these cells and reduces their proliferative capacity, resulting in bi-potential progenitors with the ability to form megakaryocytes (Meg) or erythroid (E) cells (MEPs). MEPs further differentiate along one (Meg) or another (E) lineage, the latter producing fully committed erythroid precursors (Figure 3.2).

These committed erythroid progenitors are defined by their growth potential and characteristics as assayed by *in vitro* cultures: this explains their names 'burst-forming' erythroid units (BFU-E) and 'colony-forming' erythroid units (CFU-E) (Figure 3.2). They are not morphologically recognisable but can be defined as discrete cell populations in cord blood, bone marrow and peripheral blood using combinations of cell surface markers (Figure 3.2). CFU-Es defined in these culture systems most closely correspond *in vivo* to proerythroblasts (see below), the earliest morphologically recognisable erythroid precursor, representing 20–60 per 10,000 nucleated cells in the bone marrow. Each of these differentiating cell types can be isolated and, to some extent, purified via their cell surface markers (Figure 3.2) and more recently, it has been shown that single cells from each population can be clustered and classified on the basis of their individual transcriptional and epigenetic profiles.

Over the past few years, it has emerged that a small number of key haemopoietic transcription factors play a major role in regulating the formation, differentiation, proliferation and survival of multipotent stem cells as they develop into red cells. These transcription factors may operate on their own or as members of multicomponent complexes involved in the activation and/or repression of gene expression. At present, the key transcription factors involved in the specification and maintenance of HSCs and their multipotent progenitors are complex and under intense investigation. During normal erythroid development, it is thought that GATA2 is one of many factors that initiate the erythroid programme and plays an important role in the expansion and maintenance of haematopoietic progenitors. The subsequent formation and differentiation of erythroid cells are under the control of a specific subset of key transcription factors including GATA1, FOG1, LMO2, LDB1, SCL1, E2A, MYB1, KLF1, NF-E2 and FOXO3. The most important transcription factor orchestrating changes in this programme is GATA-1 which reaches its highest levels in CFU-Es and proerythroblasts. These factors bind in various combinations to the regulatory elements that switch erythroid-specific genes on and off and thereby control gene expression during erythropoiesis. Additional, more minor roles in refining the erythroid programme are played by long non-coding RNAs and microRNAs. One emerging principle in our understanding of lineage commitment is that factors affiliated with different 'master' regulators such as GATA1 (erythroid) and PU.1 (lymphocytes and granulocytes) are both present in uncommitted progenitors, reflecting the potential of these cells to develop along alternative pathways (so-called multilineage priming). It is now known that, for example, GATA1 and PU.1 interact and cross-antagonise each other. Therefore, as cells differentiate, reinforcement of the transcriptional programme of one lineage may actively suppress that of alternative lineages.

Differentiation and terminal maturation

The earliest recognisable committed erythroid precursor in the bone marrow is the proerythroblast, a large cell (diameter 20–25 μm) with prominent nucleoli. Division of proerythroblasts leads to progressively smaller basophilic erythroblasts (16–18 μm), early polychromatic erythroblasts (10–15 μm), and finally late polychromatic/orthochromatic erythroblasts (8–10 μm). Again, each of these cells can be identified morphologically and via their cell surface markers (Figure 3.2). Early and late erythroblasts can be separated using antibodies directed against the transferrin receptor (TfR, CD71) which decreases during terminal differentiation, and glycophorin A (CD235a) which increases during maturation. Recently, alpha4-integrin (CD49d) and band 3, have been shown to further improve the resolution of the various stages of terminal erythroid differentiation. The majority

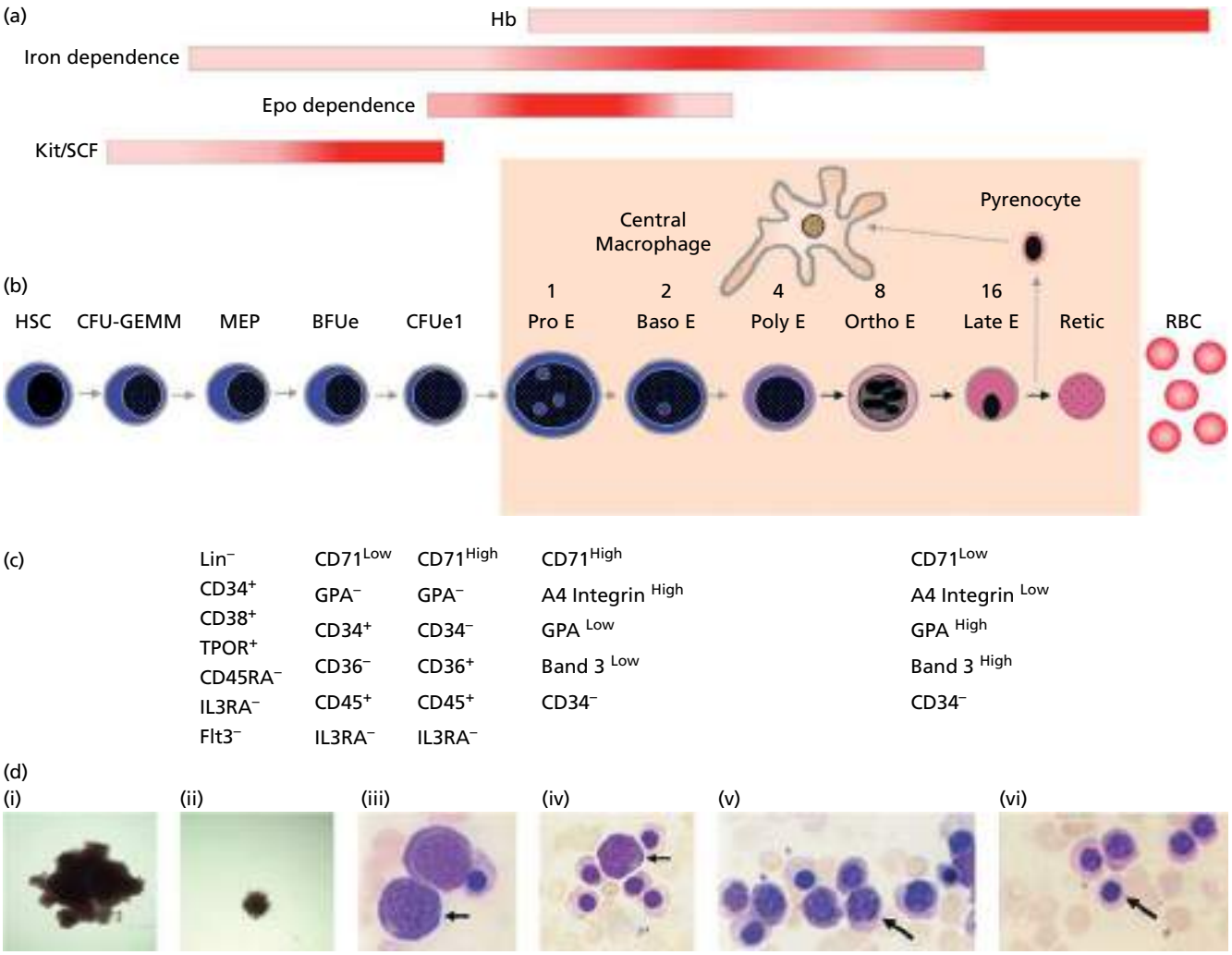

Figure 3.2 The process of erythropoiesis as cells differentiate and mature into red blood cells. (a) shows the key cytokines and processes associated with the progressive stages of erythropoiesis. (b) shows the defined cellular stages of erythropoiesis. The numbers above indicate the number of progeny arising from one proerythroblast. The transition from proerythroblast to reticulocytes occurs in a specific niche in the bone marrow (shown boxed), a process that occurs in clusters of 30 erythroblasts around a central macrophage. Small blue circles represent nucleoli. (c) shows changes in some cell surface markers at different stages of erythropoiesis. (d) photo micrographs of cells from cell cultures (i) BFUe (ii) CFUe, and arrowed cells at progressive stages of differentiation (iii) proerythroblast (iv) basophilic erythroblast (v) poly and ortho erythroblasts (vi) late erythroblasts.

(60–80%) of proerythroblasts, basophilic erythroblasts and early polychromatic erythroblasts are in active phases of the cell cycle. By contrast, late polychromatic/orthochromatic erythroblasts are postmitotic, non-dividing cells. On average, approximately 16 late erythroblasts arise from each proerythroblast although some early erythroblasts may be lost via apoptosis (Figure 3.2).

During the later stages of erythropoiesis, histones are partially released into the cytoplasm and degraded by the proteasome pathway. Eventually, extrusion of the condensed, pyknotic nucleus from orthochromatic erythroblasts gives rise to pyrenocytes (the expelled nucleus surrounded by a thin rim of cytoplasm) and immature reticulocytes (8–10 μm), which retain some mitochondria and polyribosomes. When stained with May Grunwald Giemsa, reticulocytes have a bluish tint and give rise to polychromasia in the peripheral red blood cells. When stained with new methylene blue, the cytoplasm has a characteristic reticular appearance. Reticulocytes undergo further maturation in the bone marrow for up to 48 hours before entering the circulation. Membrane remodelling occurs through a process referred to as internalisation, followed by exocytosis of the membrane and cytoplasmic fragments. Ferritin-laden vacuoles and transferrin receptors are also removed by exocytosis in clathrin-coated pits. In normal human blood, reticulocytes constitute approximately 1–2% of the total erythrocyte count. Haemoglobin synthesis

begins at the proerythroblast stage of erythropoiesis and reaches a maximum in the intermediate phases: haemoglobin continues to accumulate, even in enucleated reticulocytes containing RNA, and confers an increasingly pink colouration to erythroid cells following Wright–Giemsa staining.

Changes in the expression of key erythroid proteins during terminal differentiation

As multipotent progenitors enter terminal differentiation and the erythroid transcriptional programme becomes consolidated, the expression of many genes (~6000) is downregulated, reflecting the commitment to a single specialised lineage. By contrast ~600 genes encoding proteins central to red cell biology are upregulated. Examples include most notably haemoglobin and genes regulating the haem synthesis pathway, together with proteins of the red cell membrane including blood group antigens. There are also changes in the enzymes of the red cell energy pathways; a notable example of this is the pyruvate kinase, encoded by the *PKLR* gene. Mutations in the *PKLR* gene are found in rare cases of haemolytic anaemia.

Key among the genes characteristically upregulated during erythroid maturation are those of alpha- and beta-globin. The globin genes are first expressed in proerythroblasts and early basophilic erythroblasts, with the number of RNA transcripts reaching ~20,000 per cell in late polychromatic and orthochromatic erythroblasts. During the latest stages of erythroid maturation, the amount of RNA per cell and the rate of total protein synthesis declines, but the unusual stability of globin mRNA ensures that globin remains the predominant protein made in late erythroblasts and reticulocytes. The synthesis of globin is accurately matched with the synthesis of haem. As discussed in Chapter 11, some steps of haem synthesis occur in the cytoplasm and others in the mitochondria (see Figure 3.3). mRNAs encoding many components of the haem biosynthetic pathway (e.g. ALA synthase and porphobilinogen deaminase) are coordinately upregulated in terminal erythroid differentiation and their genes contain similar *cis*-regulatory elements activated by the same set of erythroid-specific transcription factors that activate the globin genes. Haem synthesis is in turn closely regulated by iron status (see below) via iron-responsive elements that control translation of some key enzymes in this pathway. Abnormal synthesis of haemoglobin (affecting globin, haem and iron) underlies the most frequent causes of anaemia throughout the world.

Changes in gene expression are also reflected in the cell-surface proteins present on erythroid progenitors and precursors, some of which mediate the different signalling programmes of erythroid cells as they differentiate. Differentiating erythroid cells also express cell-surface adhesion molecules (α4β1 integrin, ICAM-4 and erythroid macrophage protein EMP) which interact with equivalent molecules present on macrophages (VCAM-1, αV and erythroid macrophage protein EMP). Cell surface signalling and adhesion molecules are found at higher levels on early precursors and are lost as maturation proceeds, freeing

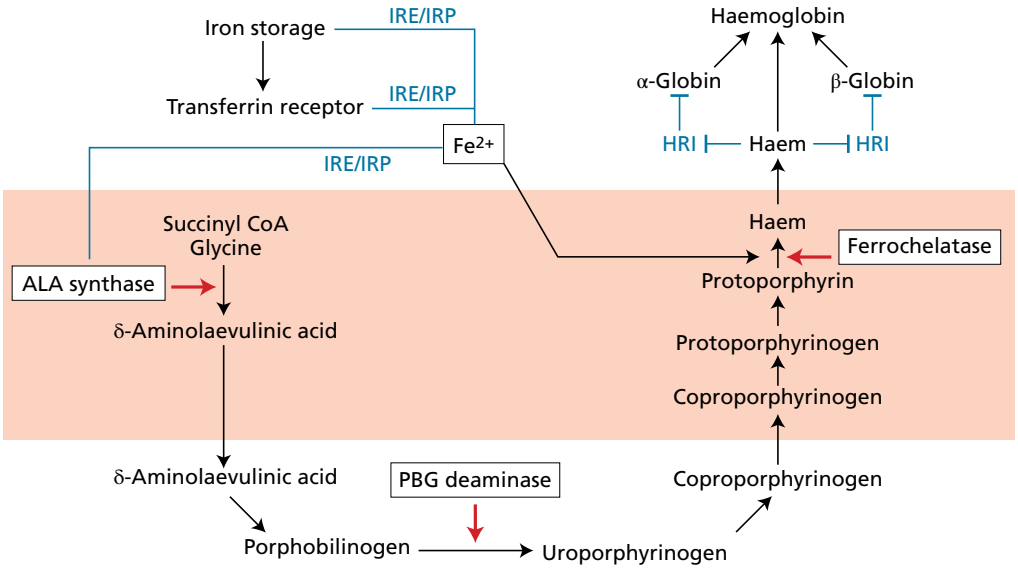

Figure 3.3 The coordinated synthesis of haem and globin to assemble haemoglobin. Some aspects of the synthetic pathway take place in the mitochondria (pink square) and others in the cytoplasm. Blue lines indicate some of the known regulatory feedback systems. Rate-limiting controls of haem synthesis are shown in black boxes. ALA, δ-aminolaevulinic acid; PBG, porphobilinogen. IRP; iron regulatory proteins. IRE; iron regulatory elements present in mRNA. HRI; Haem-regulated eIF2α kinase (HRI) controls translation by phosphorylating eIF2α.

erythroid cells from the bone marrow niche (see below) to enter the circulation. Among many changes that occur in the membranes of erythroid cells during differentiation, receptors for the key erythroid hormone erythropoietin (Epo, see below) first appear in small numbers on late BFU-Es, increasing in CFU-Es and pro-normoblasts and subsequently declining and disappearing in later erythroid precursors (Figure 3.2). Similarly, CD71 (the transferrin receptor, TfR) which allows transferrin-bound iron to be taken into the cell, is present in early haematopoietic cells but is considerably upregulated on cells that are actively synthesising haemoglobin, reaching a peak of 800,000 molecules per cell on polychromatic normoblasts. CD71 levels diminish in the late phase of terminal differentiation and the receptor is undetectable on mature erythrocytes, which no longer have a need for iron uptake.

Erythroblasts contain mitochondria and generate energy via oxidative phosphorylation. During differentiation, the number of mitochondria is reduced and mature red cells contain none but still require energy for a variety of processes. These include maintaining electrolyte gradients, synthesising glutathione, maintaining iron in its functional ferrous state, and protecting enzymes, haemoglobin and membrane proteins from oxidative denaturation. Mature red cells therefore depend on the anaerobic conversion of glucose by the Embden-Meyerhof (glycolytic) pathway for the generation and storage of high-energy phosphates.

The high levels of protein synthesis and rapid cell proliferation that characterise the erythroid compartment, even in a steady state, render these cells sensitive to perturbations in levels of the key substrates required for erythropoiesis. Among these are a variety of nutritional factors and cofactors, particularly iron (see Chapters 11 and 12), vitamin B12 and folate (Chapter 13), but also copper, manganese, cobalt, vitamin C, vitamin E, vitamin B6 (pyridoxine), thiamine, riboflavin, pantothenic acid and amino acids. Absolute or relative deficiencies of any of these factors can impair normal erythropoiesis and result in anaemia.

The erythroid niche

The control of erythropoiesis ultimately depends on integration of the internal transcriptional and epigenetic programme, modified by information delivered by cell–cell contacts, local growth factors, fine tuning of metabolism, a supply of iron and systemic hormonal signalling. Although erythropoiesis can be largely recapitulated using liquid cultures to which appropriate nutrients, cytokines and hormones have been added, it is nevertheless significantly less efficient in this context than *in vivo*, particularly in terms of proliferation and terminal enucleation, suggesting that erythropoiesis in its natural setting is modified by additional factors.

Inspection of the bone marrow architecture *in vivo* shows that maturing erythroblasts are not randomly distributed; rather, they are arranged in characteristic 'erythroid islands' made up of a central macrophage surrounded by up to 30 erythroid cells at various stages of differentiation (see Figures 3.2 and 3.4). This cellular structure is referred to as the 'erythroid niche'. Proerythroblasts enter this niche as large cells with active nuclei and mitochondria producing haem and energy. They attach to the central macrophage via a range of adhesion molecules: for example, integrins (such as α4β1) facilitate the attachment of

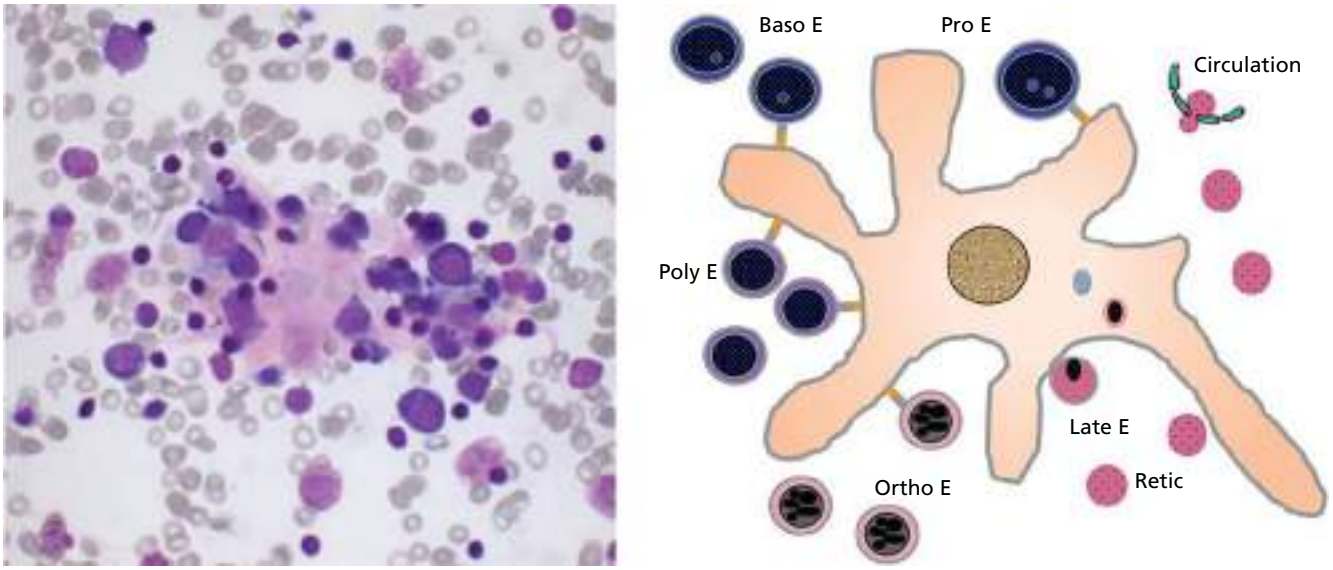

Figure 3.4 The erythroid niche. Left is a photomicrograph showing erythroid cells clustered around a central macrophage in the bone marrow. Right shows the differentiation of erythroid cells in this context. Erythroid cells attach to the central macrophage via a variety of adhesion molecules including VCAM-1, VCAM-4, α4β1integrin, αV and erythroid macrophage protein (EMP).

erythroblasts to adhesion molecules (such as VCAM-1) expressed by central macrophage, and this appears to be critical for normal red cell maturation. Soluble factors secreted by central macrophages add another layer of positive regulation within erythroblastic islands. Macrophage cytokines, including burst-promoting activity (including BMP4) and insulin-like growth factor-1 (IGF-1), induce growth of both BFU-Es and CFU-Es. Within the erythroid niche, proerythroblasts mature into later erythroblasts and, following enucleation, autophagy of mitochondria (mitophagy) also occurs. When reticulocytes exit the erythroblastic island, they are relatively small cells, without nuclei and with few mitochondria; they possess some polyribosomes and have a profoundly altered array of cell surface proteins, partly due to reassortment of cell surface molecules between the reticulocyte and pyrenocyte, enabling the release of reticulocytes from the macrophage.

The central macrophage not only provides erythropoietic signals but also plays a key role in enucleating the erythroblast. Pyrenocytes display phosphatidylserine (PS) on the surface of the cell membrane that surrounds the condensed erythroblast nucleus and this serves as a signal for macrophages to engulf and phagocytose the pyrenocyte whose nucleus is then degraded via a process involving an endonuclease (DNAse II) provided by the macrophage. It has also been suggested that the macrophage functions as a 'nurse' cell, providing iron to developing erythroblasts for haem synthesis (see below). The central macrophage may play other important roles in normal erythropoiesis. Some adhesive linkages may activate cell signalling pathways coordinating adhesion and gene expression. The juxtaposition of erythroid cells may also facilitate the action of molecules presented on cell surfaces mediating cell–cell interactions and apoptosis (see below).

It appears that the central macrophage of the erythroid niche may be a specialised cell and different from other macrophages. Central macrophages have unique properties compared to mature macrophages in other organs. For example, the central macrophage does not engulf and destroy the entire erythroid cell as splenic red pulp macrophages do. Thus, phagocytic activity of the central macrophage appears to be restricted to engulfing and processing pyrenocytes. Reticulocytes eventually enter the circulation via diapedesis through the bone marrow capillaries (Figure 3.4).

The regulation of erythropoiesis by cytokines and cell signalling

The differentiation of haematopoietic stem cells into mature blood cells is tightly regulated by the actions of both stimulatory and inhibitory cytokines. Perhaps the most important of these in erythropoiesis is erythropoietin (Epo). Erythropoietin is a 166-amino-acid 34.4-kDa glycoprotein, found in the plasma at baseline levels of 1–30 mU/mL that can be elevated 1000-fold by severe anaemia. It contains about 40% carbohydrate is rich in sialic acid residues and has a half-life of 7–8 hours in plasma. The main site of Epo production is the interstitial cells of the kidney. Under normoxic conditions, little or no Epo mRNA is detectable in the kidneys. Hypoxia results in the rapid induction of its expression such that levels may increase up to 200-fold over baseline within 30 minutes. Quite low numbers (20–50) of Epo receptor (EpoR) homodimers are present on BFU-Es, explaining the relative Epo unresponsiveness of these cells; much higher levels (~1000 EpoRs) are found on CFU-E, proerythroblasts and basophilic erythroblasts which are the main targets for Epo (Figure 3.2).

Late BFU-Es, CFU-Es and proerythroblasts all require continuous signalling via the EpoR to proliferate, differentiate and prevent programmed cell death (apoptosis). When the EpoR is bound by Epo it activates the JAK2–STAT5 pathway and Ras/MAP kinase pathways. STAT5 is a transcription factor and when activated it is translocated from the cytoplasm to the nucleus where it modulates the expression of several genes involved in the control of cell proliferation, differentiation and apoptosis. Epo signalling also activates and induces other pathways, including the phosphatidylinositol 3-kinase (PI3K) and AKT kinase pathways. The overall effect is that secondary to a hypoxia-driven rise in Epo, a population of erythroid progenitors normally destined for apoptosis during steady-state erythropoiesis receives anti-apoptotic signals, survives and expands the erythroid precursor pool. While the most important effect of Epo is to increase the number of progenitor cells, which rapidly respond by proliferating and differentiating into viable proerythroblasts, it has been suggested that Epo is also able to speed up the rate of terminal differentiation by shortening the cell cycle and maturation times of erythroblasts.

Erythropoiesis is also influenced by pathways other than Epo–EpoR. Erythroid progenitors express receptors for SCF (Stem Cell Factor), IGF-1, insulin, activin and angiotensin II. After Epo, the second most important signalling system for erythropoiesis involves SCF (also termed Kit ligand) and its receptor (c-Kit, a receptor tyrosine kinase). SCF was originally identified by its ability to stimulate proliferation of multipotent haemopoietic progenitors, but it is also effective in supporting growth of committed erythroid progenitors, acting synergistically with Epo. In addition to SCF and Epo, stimulation of the nuclear hormone receptors for dexamethasone (glucocorticoid receptor) and oestrogen (oestrogen receptor) produces sustained proliferation of erythroid progenitors. Furthermore, the nuclear hormone receptors for thyroid hormone (c-ErbA/thyroid hormone receptor), all-*trans* retinoic acid (retinoic acid receptor) and 9-*cis*-retinoic acid (RXR) can promote erythroid differentiation. Such observations are consistent with reports showing that many endocrine disorders (hypothyroidism, hypopituitarism, Addison's disease and male hypogonadism) can be associated with normochromic normocytic anaemia.

Some signalling factors including inflammatory cytokines such as tumour necrosis factor have negative effects on erythropoiesis. Recent studies have shown that a member of the transforming growth factor β superfamily (growth and differentiation factor 11, Gdf11) when bound to activin type IIB (ActRIIB) receptors, activates Smad2/3 dependent signalling to promote expansion of the committed erythroid progenitor pool, while preventing terminal maturation via an autocrine signalling loop. This pathway may be potentiated by oxidative stress but can be inhibited by activin receptor IIA ligand traps (ActIIA) such as Sotatercept or Luspatercept, which are thought to sequester Gdf11 and allow differentiation to proceed both in the steady state and under conditions of erythroid stress. Other explanations for the effects of these two drugs are also being explored. In normal erythropoiesis, Gdf11 may be transiently activated under conditions requiring an acute demand for RBCs. Gdf11 acts to rapidly expand the erythroid progenitor pool, and shortly after may become degraded or downregulated to allow for terminal maturation in response to another, as yet unidentified signal. Gdf11 appears to play an important role in the abnormal ineffective erythropoiesis seen in patients with thalassaemia and myelodysplasia where the associated anaemia may be ameliorated by administration of activin receptor ligand traps (Figure 3.5).

The regulation of apoptosis in erythropoiesis

Normal erythropoiesis requires an appropriate balance between the rate of production of erythroid cells and the rate at which immature erythroid cells undergo programmed cell death (apoptosis). Despite the efficiency of erythropoiesis, a small but significant number of early erythroid cells undergo premature intramedullary death even under normal physiological conditions. Immature erythroid precursors are sensitive to apoptotic triggering mediated by activation of both the intrinsic and extrinsic apoptotic pathways. The major extrinsic pathway controlling apoptosis of immature erythroid cells is activated by the Epo-EpoR pathway.

As discussed above, Epo signalling activates STAT5, a transcription factor that directly binds the promoters of many erythroid genes including a gene encoding the anti-apoptotic protein Bcl-XL. Bcl-XL is also regulated by the master erythroid transcription factor GATA-1 which is itself activated by Epo signalling. Bcl-XL is a key regulator of apoptosis in erythroid cells. This protein normally lies within the outer membrane of mitochondria maintaining their viability under conditions of cellular stress, thereby preventing apoptosis. In addition to increasing expression of the *Bcl-XL* gene, via the Stat5 pathway and via GATA-1, Epo may also protect erythroid cells from apoptosis by blocking caspase-dependent cleavage of Bcl-XL protein (Figure 3.6). In addition to the Epo-EpoR pathway, other cytokines such as stem cell factor (SCF) and its receptor c-Kit, also play an important role in maintaining the balance between proliferation and apoptosis during erythropoiesis (Figure 3.5).

The juxtaposition of immature and maturing erythroblasts within the erythroid niche facilitates an additional mechanism for the regulation of apoptosis. In addition to the Epo-Bcl-XL pathway, apoptosis of erythroid precursors can be regulated via the death domain receptors of the TNF superfamily such as the Fas receptor (FasR). FasR is present on the membranes of both early and late erythroid precursors. However, its activating ligand (FasL) appears only on the membranes of mature erythroblasts. Binding of FasL (on mature erythroblasts) to FasR (on immature erythroid cells)

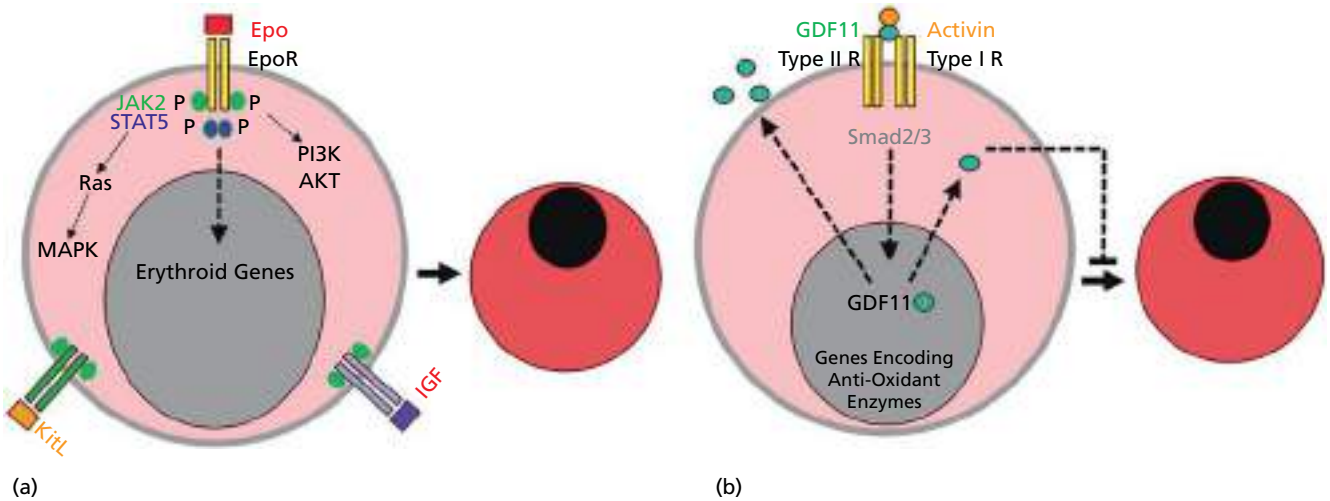

Figure 3.5 Positive and negative cytokines acting on erythroid cells. (a) positive signalling via the Epo-EpoR pathway. (b) Negative regulation of differentiation with expansion of immature erythroid cells via expression of Gdf11.

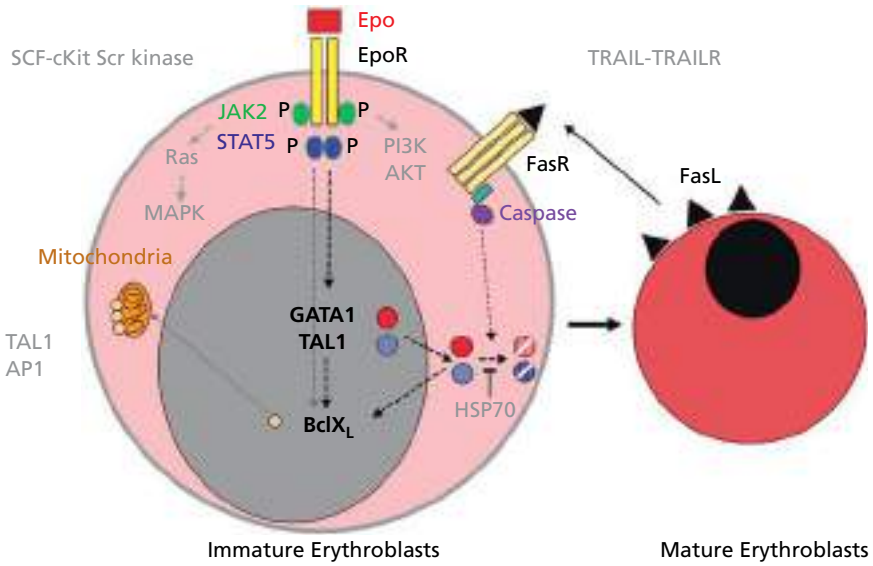

Figure 3.6 A negative feedback loop controlling the numbers of mature erythroid cells. Regulating apoptosis of immature erythroid cells and progression through terminal erythroid differentiation involves signalling via the Epo-EpoR pathway and signalling via death receptors, in this case FasR. A small beige spot in the nucleus represents Bcl-XL. Modifiers (in grey) point to pathways in addition to Epo and Fas that play important roles in regulating erythropoiesis.

activates proteolytic caspases that cleave intracellular proteins, including GATA-1. Cleavage and inactivation of GATA-1 reduces expression of Bcl-XL and promotes apoptosis in immature cells. Therefore, apoptosis only occurs in the context of adequate numbers of mature erythroid cells, in close contact, within the erythroid niche (Figure 3.4). Importantly, Fas-mediated cytotoxicity against immature Fas-positive erythroblasts is in part abrogated by high concentrations of Epo and/or SCF.

Degradation of GATA-1 appears to underlie the Fas-mediated blockade of erythroid cell maturation. However, normally, a Heat Shock Protein (HSP70) protects GATA1 from caspase cleavage during human erythroid maturation. In committed erythroid progenitors and early-stage erythroblasts, HSP70 colocalises with GATA1 and protects it from cleavage. HSP70 decreases when the FasR pathway is activated and consequently, the balance between differentiation and apoptosis of erythroblasts depends at least partly on the abundance and nuclear localisation of HSP70. In addition to FasR, other death receptors, such as TRAIL-R1 and TRAIL-R2 are also expressed on erythroid cells. Again, the expression of these two membrane receptors is higher in immature than in mature erythroblasts. Therefore, the role of the TRAIL/TRAIL-R system may be comparable to that observed for the Fas/FasL system.

Together these observations indicate that the apoptotic effects and inhibitory actions on cell differentiation elicited by Epo deprivation and/or by triggering of death receptors, direct the caspase-mediated cleavage of GATA-1, whose integrity and activity are essential for normal erythroid maturation and survival. These pathways underlie negative feedback loops in which mature erythroid cells control the numbers of earlier precursors by regulating the extent to which they undergo proliferation, differentiation or apoptosis thereby controlling the production of mature erythroid cells. These apoptotic mechanisms are frequently inappropriately activated in some pathological conditions associated with ineffective erythropoiesis and anaemia, including the thalassaemias, myelodysplasia and aplastic anaemia. In summary, erythropoiesis is driven by a balance between positive and negative feedback mechanisms operating within the erythroid niche, involving both cell–cell interactions and soluble factors.

The coordination of erythropoiesis and iron metabolism

The terminal stages of erythropoiesis are dedicated to producing 1–2 billion red cells containing 7–8 g of haemoglobin each day and this process requires ~2–3 × 10^{15} iron atoms every second. Therefore, the production of red blood cells requires balanced crosstalk with iron metabolism. The pathways that maintain appropriate levels of iron affect erythropoiesis and *vice-versa*. While the early stages of erythropoiesis are predominantly controlled by the Epo-EpoR pathway, the later stages of erythropoiesis are regulated by the availability of iron. Iron metabolism is primarily regulated by the hormones hepcidin and erythroferrone. Within erythroid cells a second system involving iron regulatory proteins and

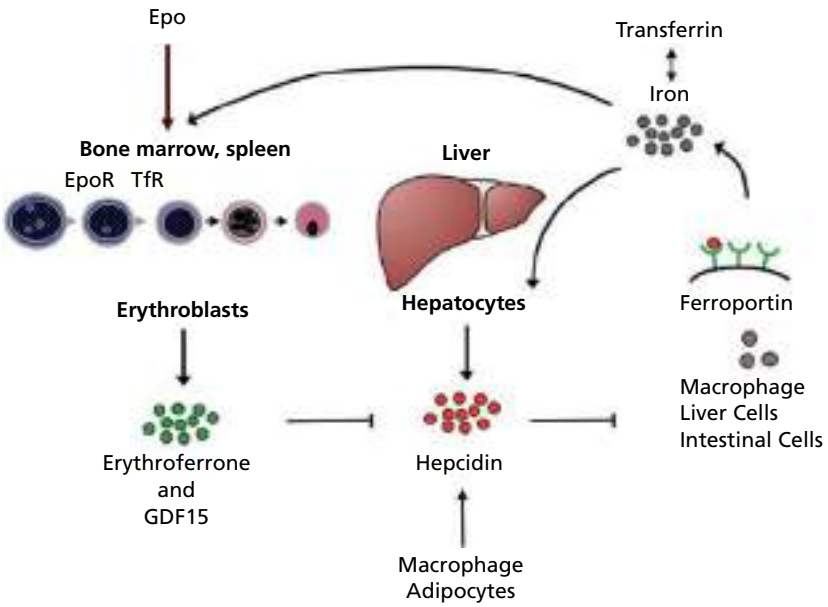

Figure 3.7 Regulation of erythropoiesis and iron metabolism. The major hormones controlling iron metabolism are hepcidin and erythroferrone. These hormones link the process of erythropoiesis and the availability of iron.

iron regulatory elements (IRP/IRE) responds to fluctuating iron concentrations, affecting translation of key iron import and export genes. The details of iron metabolism are discussed in Chapter 11 (Figure 3.7).

Hepatocytes directly sense transferrin-bound iron, which in turn stimulates production of hepcidin to limit the release of iron when it is readily available in the circulation. Under iron-replete conditions, hepcidin levels in the blood are high, which signals to iron-storing organs to suppress the release of iron to the plasma. Hepcidin directly binds and induces the endocytosis and proteolysis of ferroportin, a transmembrane exporter of iron found mainly in intestinal cells, macrophages and hepatocytes; the main reservoirs of iron in the body. By contrast, when more iron is required as a result of increased erythropoiesis, differentiating erythroblasts produce the hormone erythroferrone (an Epo-responsive gene), which suppresses hepcidin production. In turn, ferroportin accumulates on iron reservoir cells leading to increased plasma iron concentrations to support haem synthesis for the production of haemoglobin. An additional and important level of control occurs via translation of mRNAs encoding the globin chains and proteins involved in iron metabolism via iron regulatory proteins (IRPs) which bind iron regulatory elements present in mRNA (IREs) and, haem-regulated eIF2α kinase (HRI) which controls translation by phosphorylating eIF2α (e.g. see Figure 3.3).

In many human diseases, the crosstalk between erythropoiesis and iron metabolism goes awry, particularly in the presence of ineffective erythropoiesis when iron may accumulate in a wide variety of tissues causing associated morbidity. This is particularly seen in patients with chronic haemolytic anaemias and thalassaemia. In chronic inflammatory disorders, cytokines drive over-production of hepcidin that in turn limits iron supply for erythropoiesis, contributing to the anaemia of chronic disease.

The control of erythropoiesis via the oxygen-sensing system

Erythropoiesis must be sensitive to changes in the levels of oxygen and varying physiological demands. This regulation hinges on sensing hypoxia and control over the number of erythroblasts and red cells. The most important mediator of this response is the transcription factor HIF (hypoxia-inducible factor). HIF acts as a dimer consisting of HIF-1α and HIF-1β which activates genes influencing adaptive responses to hypoxia (Figure 3.8) in a wide variety of tissues. In the context of normal oxygenation, HIF-1α is modified by prolyl and asparaginyl hydroxylases, providing a binding site for vHL (the von Hippel Lindau factor – see Figure 3.8), which ubiquitinates HIF thereby targeting it for degradation by the proteasome. At low oxygen tension, hydroxylation of HIF-1α cannot occur and as a consequence, vHL cannot bind and ubiquitinate HIF-1α, the level of which is thereby greatly increased. Stabilised HIF-1αβ then activates genes encoding Epo in the kidney, enzymes of the glycolytic pathway, TfR and VEGF. In rare cases of inherited polycythaemia, constitutive mutations in HIF or vHL result in deregulated oxygen sensing and an erythropoietic drive in the absence of hypoxia (Chapter 35).

Increased expression of Epo is accomplished through a hypoxia-response element (HRE) at the 3′-end of the Epo gene,

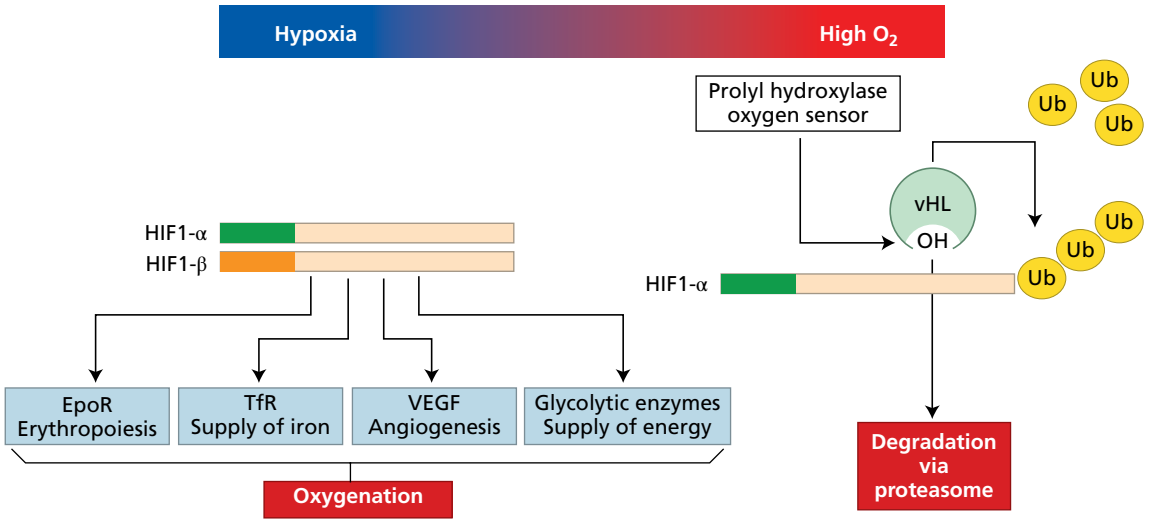

Figure 3.8 The oxygen-sensing system. Under normoxic conditions, HIF-1α is hydroxylated and is thereby targeted by vHL for ubiquitination and degradation by the proteosome. Under hypoxic conditions, this no longer occurs and the level of HIF-1α rises and dimerises with HIF-1β to form a transcription factor that activates oxygen-responsive genes in the pathways indicated. Although this response is present in all cell types, upregulation of Epo, erythroferrone, genes controlling iron metabolism and the glycolytic pathway are of particular importance in erythropoiesis. Ub, ubiquitination; vHL, von Hippel–Lindau protein; HIF, hypoxia-inducible factor. vHL is an E3 ubiquitin ligase.

which increases its expression. Under hypoxic conditions, HIF1-αβ is stabilised and can bind to the HRE of the *Epo* gene (and other hypoxia-sensitive genes), to increase transcription and therefore increase serum Epo levels. Linking Epo production to tissue oxygenation ensures that when there is reduced ambient oxygen tension, blood loss, or shortened red cell survival, the level of Epo and other hypoxia-responsive genes increase, stimulating red cell production and ultimately increasing oxygen delivery.

The mature red cell, senescence and clearance

After 2–3 days in circulation, the red cell lacks the machinery for protein synthesis and the organelles for complex cellular processes. In essence, it becomes a highly concentrated solution of haemoglobin enclosed within a complex membrane that maintains the shape and integrity of the biconcave disc (7–8 μm), which we recognise as the mature red blood cell. In adults, such cells survive for approximately 120 days, travelling 250 km throughout the circulation whilst exposed to repeated membrane deformation and mechanical stress. In addition, while transporting high concentrations of oxygen, red cells experience chemical damage from oxidative species and free radicals. Since mature red cells lack the capacity to synthesise new cellular components, their ability to maintain cellular integrity in the face of these stresses becomes compromised with age. Although ageing red cells cannot be distinguished morphologically, characteristic biochemical features include a reduction in sialic acid, cholesterol, phospholipids and a variety of membrane proteins and a reduction in enzymatic activity, including enzymes of the glycolytic pathway. There is a loss of haemoglobin and an increase in its glycation (measured as HbA1C). Another feature of ageing red cells is microvesiculation; although this provides an effective means of removing damaged or ineffective red cell components, it results in the continual loss of small fragments of the red cell. Together, loss of haemoglobin and red cell membrane ultimately produces cells that are denser, with a higher mean corpuscular haemoglobin concentration (MCHC) and less deformable than their younger counterparts. Such aged red cells are also compromised with respect to their ability to efficiently perform ion transport.

As well as these changes, specific cues to clearance of the aged red cells from the circulation are thought to exist. Phosphatidylserine and phosphatidylethanolamine are key constituents of the red cell membrane that are normally confined to the inner aspect of the lipid bilayer. This asymmetry is maintained by an ATP-dependent aminophospholipid translocase. In aged red cells, phosphatidylserine is found in the outer membrane where it is able to bind to macrophages in the liver and spleen and prompt erythrophagocytosis. Once directed to

macrophages, these effete red blood cells undergo phagocytosis and the red cell components, including the iron from its haem groups, are recycled for subsequent red cell synthesis.

Assessing erythropoiesis

Erythropoiesis is disturbed in a wide range of primary haematological conditions and, to some extent, in almost all multisystem diseases. Initial assessment can be logically based on the successive stages of erythropoiesis set out above. In current clinical practice, erythropoiesis can be most simply assessed by examination of the full blood count and red cell indices, the reticulocyte count and examination of the peripheral blood film and bone marrow morphology. The specific tests used to diagnose the inherited or acquired disorders that may perturb each phase of erythropoiesis are highlighted in the relevant chapters as each anaemia is discussed in detail.

Initial assessment of erythropoiesis can be made from the red cell indices included in the full blood count. Anaemia is said to occur when the level of haemoglobin (g/L) and haematocrit fall below the normal ranges which vary depending on age and sex. The level of haemoglobin reflects the number of red cells/mL of blood (RBC) and their mean corpuscular haemoglobin content (MCH pg). It should be noted that individuals with a low MCH may be anaemic despite a raised red blood cell count. The MCH is largely dependent on the synthesis of haemoglobin and is commonly reduced in iron deficiency, defects of the haem synthesis pathway (e.g. sideroblastic anaemias) or in disorders of globin chain synthesis (haemoglobinopathies). The size of the red cells depends to some extent on the number of cell divisions in the terminal stages of differentiation. Thus, the mean corpuscular volume (MCV fl) is reduced when cells go through an extra division in terminal erythropoiesis (iron deficiency and thalassaemia) and increased when cells go through fewer cell divisions, often associated with dyserythropoiesis (e.g. B12 and folate deficiency, myelodysplasia, congenital dyserythropoietic anaemia). Additional clues to the pathogenesis of the anaemia can be derived from inspection of the red cell morphology on a peripheral blood film (Chapters 11, 13 and 56).

A second critical assessment of erythroid activity can be obtained (when appropriate) from an examination of the bone marrow. The number and ratio of erythroid compared to myeloid precursors in the marrow is normally about 1:4 but may vary within a very broad normal range. When erythropoiesis is stimulated, the ratio of erythroid to myeloid precursors will increase and this may be represented by normal precursors or dyserythropoietic precursors identified via asynchrony between haemoglobinisation and nuclear maturity. In addition, dyserythropoiesis may be accompanied by abnormal nuclei and sometimes binuclearity in erythroid cells. These changes in erythropoiesis are also reflected in the reticulocyte count. When erythropoiesis responds appropriately to anaemia (e.g. caused by bleeding or haemolysis) in the presence of adequate iron stores, the reticulocyte count (normally 0.5–2.0% or $25–75 \times 10^9$/L) increases in proportion to the degree of anaemia. By contrast, in the presence of bone marrow failure or significant dyserythropoiesis, the reticulocyte count is inappropriately low for the associated degree of anaemia. More recently, it has become possible to specifically measure the haemoglobin content of reticulocytes. This provides an indirect measurement of the functional iron available for new red blood cell production over the previous 3–4 days, providing a guide to the quality of erythropoiesis with respect to available iron.

In addition, when iron stores are adequate and available, measuring the level of soluble TfR (normal range 5.0 ± 1.0 mg/mL) also provides a good guide to the total level of erythropoiesis. Soluble TfR is a truncated form of the receptor that circulates in a complex with transferrin. Erythroblasts rather than reticulocytes are the main source of soluble TfR, and the levels of TfR are increased when erythropoiesis is stimulated and decreased when erythropoiesis is diminished. The interpretation of soluble TfR levels is complicated in iron deficiency as this condition independently raises the level of soluble TfR (Figure 3.9).

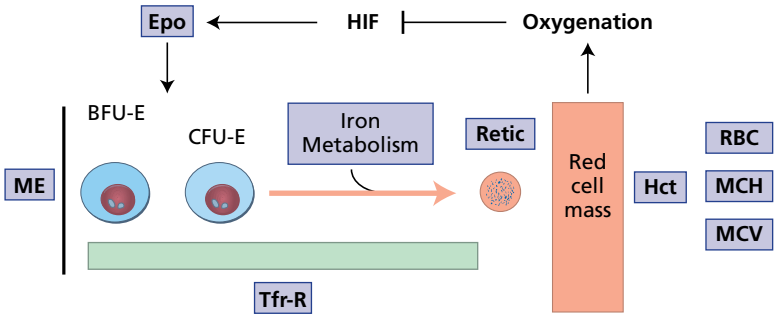

Figure 3.9 Summary of the regulation of erythropoiesis. Note the key points for assessment boxed in grey. ME denotes assessment of the myeloid/erythroid ratio in the bone marrow. Epo, erythropoietin; Hct, haematocrit; MCH, the mean cell haemoglobin; MCV, the mean cell volume; RBC, red blood cell count; Retic, Reticulocyte count; Tfr-R, soluble transferrin receptor.

If the red cell mass is appropriate to meet the demands for oxygenation, then Epo production will be suppressed and the serum level will be in the normal range (~10–30 mU/mL in adults). If there is inadequate oxygenation, the level of Epo will generally be raised in proportion to the degree of anaemia (e.g. up to 3–10 U/mL after severe blood loss) unless there is impairment of Epo production (e.g. chronic kidney disease, anaemia of chronic disease). For any given degree of anaemia, the level of Epo in the blood varies depending on the underlying pathology. For example, levels tend to be very high in aplastic anaemia and less than anticipated in thalassaemia. This may reflect the different numbers of precursors in the marrow that are able to bind available Epo molecules, thus altering the number of free Epo molecules that are measured.

The close crosstalk between iron metabolism and erythropoiesis means that erythropoiesis may also be affected by disorders primarily affecting iron metabolism, most often iron deficiency or the anaemia of chronic inflammatory diseases. Therefore, any assessment of erythropoiesis also needs to take into account the availability of iron. The assessment of iron status and metabolism by measuring serum iron, transferrin, iron saturation, ferritin, hepcidin and erythroferrone, is set out in Chapter 11.

Concluding remarks

The process of erythropoiesis needs to be tightly controlled to ensure adequate oxygen delivery to the tissues across a range of physiological and pathological conditions. An appreciation of the mechanisms of erythroid specification, maturation and feedback has already led to an improved understanding of the pathophysiology of many inherited and acquired anaemias, and provides a framework for the logical investigation and treatment of anaemia in the clinical setting.

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CHAPTER 4

Lymphocyte development and function

4

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Introduction

The immune system protects against infection and cell changes that can lead to the development of malignancies or autoimmune diseases. It can be broadly divided into two functional arms, also known as the *innate* and *adaptive* immune arms. In reality, these two arms overlap and there are extensive interactions between the two. The innate immune system is largely responsible for the initial rapid response to infection and is mediated by cells such as neutrophils, macrophages and natural killer (NK) cells. The adaptive immunity arm comprises of T and B cells, which responds upon recognition of specific molecules (antigen, for B cells) or when the molecules are processed and short regions of the antigen (peptide) are presented by antigen-presenting cells such as dendritic cells (DC) to T cells.

Cells of the immune system are derived from self-renewing haematopoietic stem cells in the bone marrow. There are exceptions, such as tissue-resident macrophages which are formed during early fetal development in the yolk sac. The haematopoietic stem cell gives rise to common lymphoid or myeloid progenitors. Differentiation of the common lymphoid progenitor gives rise to B cells, T cells, NK cells, NKT cells and DC. The latter can also be derived from a myeloid progenitor.

Most *B cells* are generated from within the bone marrow, the 'B' in their name referring to an obscure avian structure called the bursa of Fabricius in which the B cells of birds develop. Naive mature B cells enter the lymphoid circulation, but, if triggered by antigen in the periphery, a proportion of cells will return to the bone marrow as long-lived plasma cells that secrete immunoglobulin. B lymphocytes are the precursors of antibody-producing cells. Each B cell produces, and expresses on its surface, immunoglobulin with a distinct specificity for antigen. The specificity of the immunoglobulin is determined by the way the immunoglobulin variable-region genes are rearranged during B lymphopoiesis. B cells that bind antigen through their surface immunoglobulin have to obtain accessory signals if they are to proliferate and differentiate into antibody-secreting cells. These can be provided by helper T cells, which recognise antigen that has been taken up and presented by the B cell.

T cells are generated in the thymus following the migration of prothymocytes from bone marrow to thymus followed by selection of thymocyte precursors. Over 95% of thymocytes die in the thymus, but the minority population emerges from the thymus as single-positive $CD4^+$ or $CD8^+$ T cells and enters the lymphoid system as naive precursors that can survive for many years. T cells have extremely diverse functions including: (i) providing signals that help induce T cells and B cells to proliferate and differentiate, (ii) specifically deleting virally infected cells or foreign cells, (iii) activating macrophages to enhance cellular cytotoxicity and (iv) regulation of established immune responses.

NK cells similarly appear to develop from within the environment of the bone marrow. They are able to kill cells that fail to express major histocompatibility complex (MHC) class I molecules on their surface or that express molecules associated with cellular stress.

The bone marrow and thymus are therefore the sites of lymphocyte development and are known as the *primary lymphoid organs*. However, immune responses are initiated when lymphocytes encounter antigen and this occurs primarily in *secondary lymphoid tissues* such as lymph nodes and the spleen.

Lymphocytes circulate around the body tissues via the blood and lymphatic vasculature. Lymphatic vessels drain extravascular spaces and lymph nodes are collections of lymphoid tissue in

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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lymphatic vessels, which are organised to optimise encounters between lymphocytes and antigen. Afferent lymph drains into the lymph nodes, bringing circulating lymphocytes and populations of antigen-loaded dendritic cells from regional tissue. Efferent lymph returns lymphocytes to the bloodstream, where naive cells continue this circulatory pattern in a continuing quest for antigenic encounter. Antigen-experienced lymphocytes migrate to a variety of tissues in order to mediate their effector functions. The pattern of homing is largely determined by the chemokine receptor profile on the lymphocyte.

In this chapter, we will discuss the development and function of each of these lymphocyte populations and their relevance in the context of disease and therapy.

B cells

Development

B-cell production starts in the fetal liver at the end of the first trimester of pregnancy and ceases at this site later in pregnancy. Subsequently, B cells are also produced in the bone marrow, and production in this tissue continues throughout life such that approximately 2% of adult marrow mononuclear cells are B-cell progenitors.

The transcription factors paired box protein 5 (Pax5), E box binding protein 2A (E2A) and early B-cell factor (EBF1) are critical in driving the differentiation of *common lymphoid progenitors* into early B cells. Mice lacking any one of these transcription factors have impaired B-cell development.

The development of B cells can be divided into *antigen-independent* and *antigen-dependent* phases (Figure 4.1). In the antigen-independent phase, the transition of early B cells from *pre-pro-B* into *pro-B* and then *pre-B* cells are marked by variable of expression of the following cell surface markers: CD34, CD19, CD10 and deoxynucleotidyltransferase (Tdt), as well as changes in surface immunoglobulin expression and rearrangement. Heavy-chain gene rearrangement precedes light-chain gene arrangement. The first rearrangements to occur are in the joining of one of the J segments to one D segment, with the looping out of the intervening sequences. If this is successful, the rearranged V–D–J sequence will be transcribed together with the genes encoding the μ constant region. These processes are mediated by RAG-1/RAG-2 enzymes, which catalyse double-strand DNA breaks at specific regions. Cleaved DNA ends are rejoined in a non-homologous fashion by DNA protein kinases. Translation of this transcript results in the cell expressing a cytoplasmic μ heavy chain (pre-B-cell receptor, pre-BCR), and transition of the cell from a *pro-B cell* to a *pre-B cell*. Only one functional VDJ rearrangement is made by each B cell and this *allelic exclusion* of antigen receptor genes means that only one functional antigen-binding protein is expressed on the surface of the cell. Cells that fail to make a single functional rearrangement die by apoptosis. The enzyme, TdT, is expressed during variable-region gene rearrangement and catalyses the addition of random nucleotides at the junctions of V–D–J and D–J to further expand the antibody specificity repertoire. The enzyme is expressed both in pro-B cells in the marrow and in cortical thymocytes during *TCR* gene rearrangement.

Each B cell expresses only a single light chain and the ratio of κ-expressing B cells to λ-expressing B cells is close to 60:40. Disturbance of the κ/λ B-cell ratio is a reliable indicator of the presence of a neoplastic clone. Intact heavy chains can be exported to the surface of a B cell only if they are complexed with light chain or surrogate light chain. This does not apply to free light chains, which are able to pass out of B cells and plasma cells. Physiological light chain production is always greater than that required to complex all the available heavy chain, and measurement of serum-free light chain is a useful tool in the management of paraproteinaemia. The heavy chains that are secreted in heavy chain disease are able to leave the cell only because they are truncated heavy chains.

The next step in B-cell development is the maturation of the pre-BCR into a BCR. This requires effective pairing of rearranged light changes with rearranged heavy chains and signifies the transition of a pre-BCR cell into an immature B cell. Here, the BCR consists of an antigen-detecting component (surface immunoglobulin M (IgM)) and a signalling component similar to the pre-BCR (Ig-α and Ig-β). VDJ recombination produces a diverse antibody repertoire, essential for host defence, but a drawback is that the specificity is generated randomly and so some of the Ig will be reactive against self-antigens. As a result, tolerance mechanisms such as *anergy* and *receptor editing* are essential. *Anergy* is a process whereby self-tolerance is generated through reduced BCR expression and signalling, whilst *receptor editing* is a process whereby the RAG enzymes continue to catalyse light chain rearrangement until a non-self-reactive BCR results.

In the *antigen-dependent phase*, the non-autoreactive, naive and *immature B cell* (also known as a *transitional B cell*) then migrates into the peripheral blood and then localises into the follicular regions of the secondary lymphoid organs (marrow, lymph nodes) where they are termed *follicular B cells*. Within the follicles of the spleen or lymph nodes, the naive follicular B cells will encounter cognate antigen complexed on the surface of complement receptors of primary *follicular dendritic cells* (FDC) and then migrate to the T:B cell border. B cells can also be activated by soluble antigen but the affinity of the antigen for the BCR is low. In contrast, membrane-bound antigens are able to tether B cells more strongly and enhance BCR signalling through co-stimulation by other surface receptors.

In the T:B cell border, B cells undergo intense proliferation to generate *centroblasts* and form *germinal centres* (GC). B-cell lymphoma 6 (BCL-6) is vital in driving B-cell proliferation and suppressing differentiation. GC formation is critical for the development of secondary immune responses and immunologic memory. Within the GC, two distinct processes occur at the immunoglobulin loci, *somatic hypermutation* and *class*

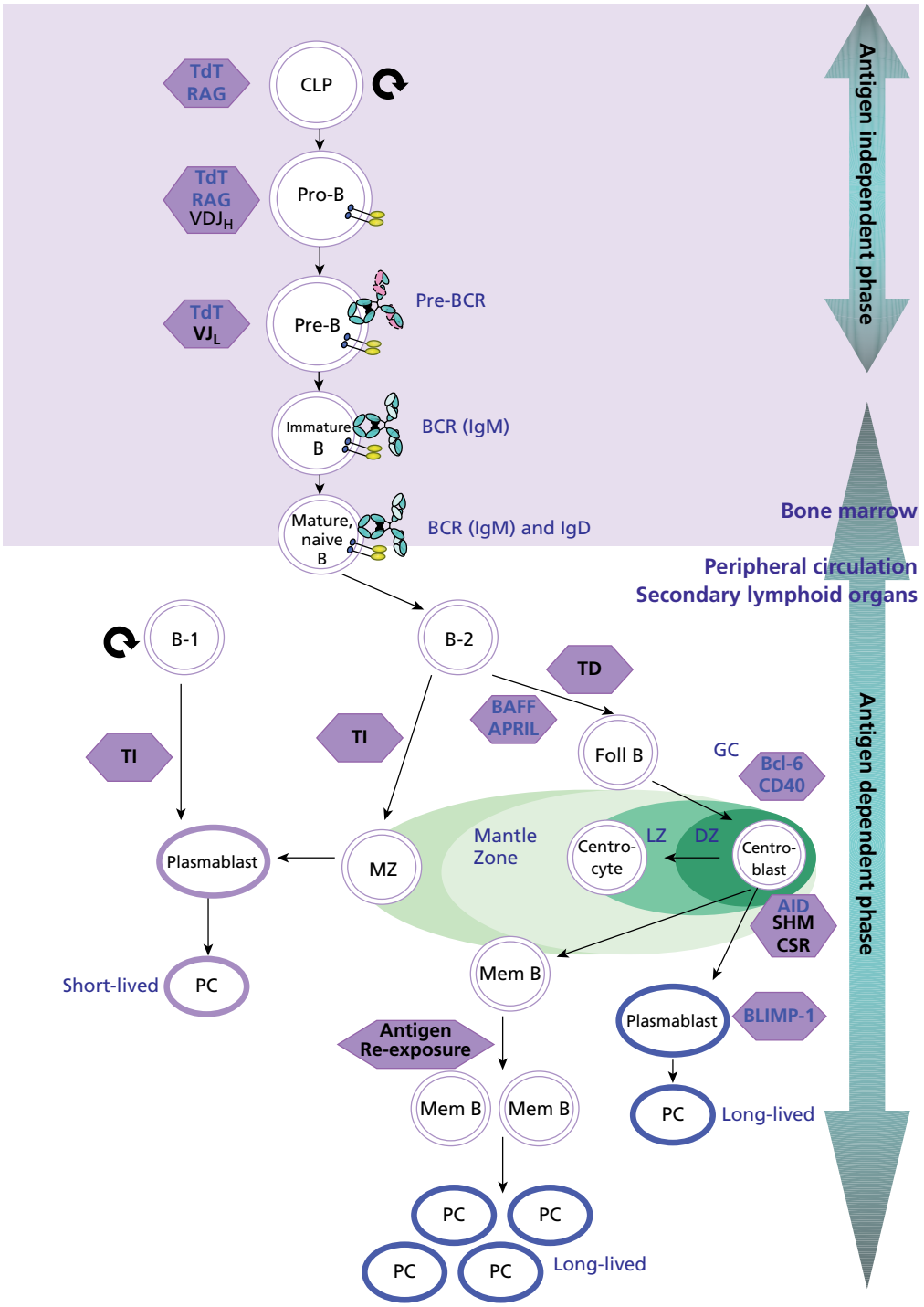

Figure 4.1 The key transcription factors and steps in B-cell development are summarised here. The antigen-independent phase of B-cell development takes place in the bone marrow. During this phase, mature but naïve B cells expressing BCR are produced. The cells then enter the peripheral circulation and upon antigen-encounter, are stimulated via T-cell independent (TI) or T-cell dependent (TD) pathways. TD-stimulated cells migrate to the germinal centre (GC) where somatic hypermutation (SHM) and class switch recombination (CSR) takes place. Proliferating and non-proliferating B-cells are known as centroblasts and centrocytes, respectively. Centrocytes will apoptose, unless they receive rescue signals from CD40-CD40L interaction on T cells, and further differentiate into either plasma cells (under control of Blimp-1) or memory B-cells. Self-renewing B-1 cells and marginal zone (MZ) B cells are stimulated via TI pathways and produce a rapid but transient Ab response. CLP, common lymphoid precursor; mem B, memory B cell; PC, plasma cell.

switch recombination. In somatic hypermutation, activation-induced deaminase (AID) catalyses the deamination of cytidine residues in the Ig variable gene, leading to the introduction of non-random point mutations, thereby increasing the affinity of the antigen-binding domain of the Ig. For class switch recombination, AID also induces double-strand DNA breaks within guanine-rich tandem repeats upstream of each heavy chain constant region leading to recombination in a non-homologous manner. This allows a switch between the variable and constant regions, thereby allowing the Ig to switch from IgM and IgD to IgG, IgA or IgE to vary its function.

Mutated centroblasts then exit cell cycle to become centrocytes and migrate to the edge of the follicle to form the morphologically described light zone. In order to achieve sufficient numbers of high-affinity mutations per B cell, centrocytes enter cell cycle and undergo somatic hypermutation repeatedly. Cells that express high-affinity Ig receptors are selected for survival, in a process called *affinity maturation*. A multitude of interdependent factors are involved in the germinal centre reaction, with the crucial cellular components being the follicular DC and the follicular helper CD4 T cells (Tfh).

Despite the fundamental need for VDJ recombination, somatic hypermutation and class switch recombination, errors during these processes are potentially pathological. Reciprocal chromosomal translocations that juxtapose a proto-oncogene next to an Ig regulatory locus are one of the hallmarks of B-cell lymphomas. Examples of these include c-myc and BCL-6 in aggressive B-cell lymphomas, BCL-2 in follicular lymphoma and BCL-1 which produces cyclin-D1 overexpression in mantle cell lymphoma.

The naive B cells can also be activated on antigen encounter through a T-cell-independent pathway. The T-cell independent pathway is a relatively weak and rapid response that serves to provide a transient protective immune response and results in the production of short-lived, IgM-secreting *plasmablasts*.

Antibodies

Antibodies are immunoglobulins produced by terminally differentiated B cells also known as plasma cells. Each plasma cell produces antibodies of identical specificity for an antigen (*monoclonal antibodies*). However, when B cells are stimulated by an antigen, different B cells recognise and respond to the antigen, and undergo expansion as described above, to produce a diverse antibody repertoire, or *polyclonal antibodies*. This is in contrast to the clonal populations found in B-cell malignancies, where the B cells or plasma cells express or secrete monoclonal antibodies.

The basic structure of a monomeric antibody molecule is a Y-shaped heterodimeric glycoprotein comprising of two large identical polypeptides (heavy chain, ~55 kDa) and two smaller identical polypeptides (light chain, ~22 kDa). (Figure 4.2) The light and heavy chains are held together by disulfide (S–S) bonds. Within each chain are variable and constant regions as defined by the diversity of the amino acid sequence. The antigen-binding surface of each antibody is formed by hypervariable regions called *complementarity-determining regions* (CDR) within the variable regions of the heavy and light chains.

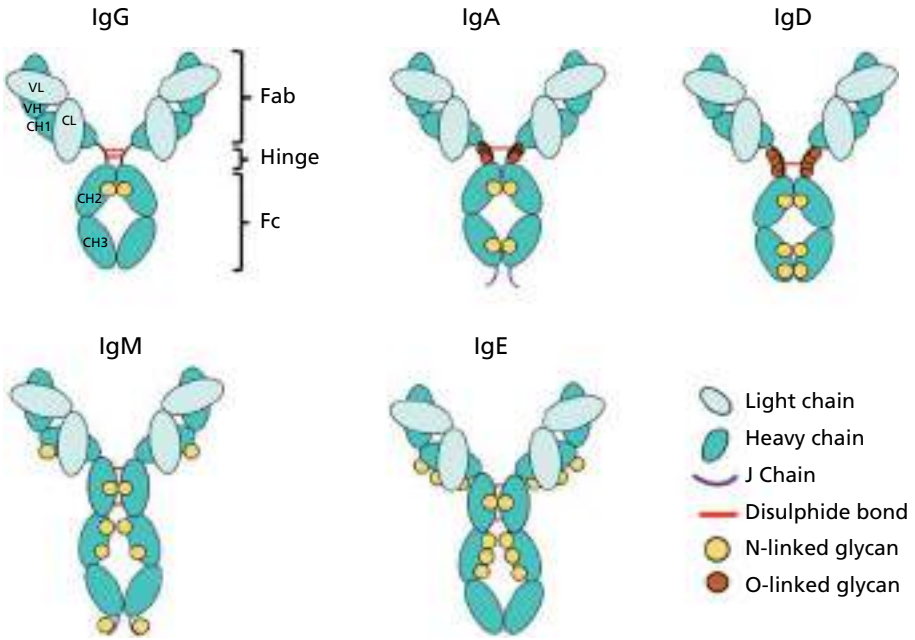

Figure 4.2 Structures of monomeric immunoglobulin molecules are shown. IgA and IgM exist as dimers (10%) and pentamers, respectively via the J chain, a 137 amino acid residue polypeptide.

There are two distinct types of light chain, kappa (κ) and lambda (λ), which are common to all immunoglobulins. Either of these chains may combine with any heavy chain, but in any one immunoglobulin molecule both light chains are identical. κ chains occur in about 65% and λ chains in about 35% of the normal immunoglobulins in each class. There are five types of heavy chains based on the amino acid sequence of the constant region (γ, α, μ, δ and ε), thus producing five classes or isotypes of immunoglobulins: IgM, IgG, IgA, IgD and IgE, respectively. IgG molecules can be further subdivided into IgG1, IgG2, IgG3 and IgG4, and IgA into IgA1 and IgA2 based on subtle amino acid sequence differences. The isotype and subclass variation allow the antibody to mediate different effector functions.

There is preferential active transport of IgG1 relative to the other IgG subclasses. As only IgG passes the placental barrier, only IgG blood group antibodies can cause haemolytic disease of the fetus and newborn (HDFN) and only IgG1 and IgG3 will mediate significant immune red cell destruction. The IgG level in cord blood will be much the same as the level in the mother. Passively transferred maternal IgG gradually disappears from the infant after birth and is almost gone by 3 months of age. The serum of newborn infants contains a small amount of IgM of fetal origin and almost no IgA; the production of IgA and IgG starts at about 1–2 months of age.

Papain can split the basic immunoglobulin molecule into three fragments at a site near the S–S bonds that hold the heavy chains together. One fragment contains the C-termini of the heavy chains and is called the *Fc (Fragment, crystallisable) fragment*. The other two are called *Fab (Fragment, antigen binding) fragments*, each of which consists of the N-terminus of the heavy chain and the whole of the light chain, and contains the antigen-binding site of the molecule.

The Fc mediates the antibodies' effector functions by binding to Fc receptors or complement components such as C1q. Essential to Fc function are sugar (glycan) domains which are linked to the heavy chains. The glycans are important for maintaining the Fc-heavy chains in an open confirmation, required for optimal binding with Fcγ receptors. As such, modification of these sugars, such as through defucosylation leads to improved formation of non-covalent interactions and subsequent increased binding affinity with this activatory Fcγ receptor. The CD20 monoclonal antibody obinutuzumab, used in follicular lymphoma, is an example of a defucosylated antibody.

Antibody function

Antibodies are multifunctional. They perform a variety of functions in addition to binding antigen, depending on their isotype. Most of these additional functions reside in the Fc fragment and will be discussed in turn.

Fc-Fc receptor-mediated interactions

After binding to antigen, the Fc domain of the antibody binds to *Fc receptors* expressed on haematopoietic cells. This is one of the main mechanisms by which the biological activity of antibodies is mediated.

There are multiple classes of Fc receptors to complement antibody isotype, e.g. IgG antibodies bind to FcγR, IgE to FcεR and IgA to FcαR and Fcα/μR, and IgM to Fcα/μR. FcγR can be further subdivided into activatory or inhibitory receptors. FcγRIIb is the sole inhibitory receptor, whilst there are multiple activatory FcγR (FcγRI, FcγRIIa and FcγRIIIa). These receptors are variably expressed in haematopoietic cells and engage the different IgG subclasses with different affinity. As a general rule, activatory FcγR promotes activation of the cell it is expressed on, whereas inhibitory FcγRIIb has the opposite effect. Thus, the balance of engagement between inhibitory and activatory FcγR engagement on a cell determines the eventual response triggered.

Two key mechanisms triggered by Fc-FcγR interaction are *antibody-dependent cellular cytotoxicity* (ADCC) and *antibody-dependent cellular phagocytosis* (ADCP). In ADCC, the antibody forms a bridge between the antigen-expressing cell and the FcγR-expressing NK cell, causing activation of the NK cell and subsequent release of cytotoxic molecules such as perforin and granzyme which lyse the target cell. Similarly, in ADCP, the antibody links the antigen-expressing cell with an FcγR-expression phagocyte such as a macrophage, which leads to cellular activation, phagocytosis and eventually lysis of the target cell. These two are mechanisms, in particular ADCP, underpin the anti-tumour efficacy of monoclonal antibodies such as anti-CD20, rituximab and obinutuzumab.

Complement fixation

The *classical pathway* of the complement system is initiated when antigen-bound antibody of particular isotype binds to the first complement component, C1. (Figure 4.3) IgM, IgG1, IgG2 and IgG3 but not IgG4 or IgA, activate the classical pathway. C1 is a complex of three protein molecules, C1q, C1r and C1s, and activation of this pathway requires multiglobular C1q to bind to at least two juxtaposed Fc regions, thus explaining why pentameric IgG is more effective at activating complement than monomeric IgG1. The C1 complex further activates C4 and C2, generating C4b2b, known as C3 convertase. The cell-bound C4b2b can optimally activate several thousand C3 molecules by splitting them into C3a and C3b. C3b molecules act as an opsonin by binding covalently to the target cell, thereby targeting it for destruction by C3b receptor expressing phagocytes. The active phase of C3 is transient because C3b is rapidly degraded by an enzyme (C3b inactivator or factor I) and its accelerator (βIH or factor H). C4b2b3b will 'trigger' the fixation of C5, C6, C7, C8 and C9, leading to the formation of

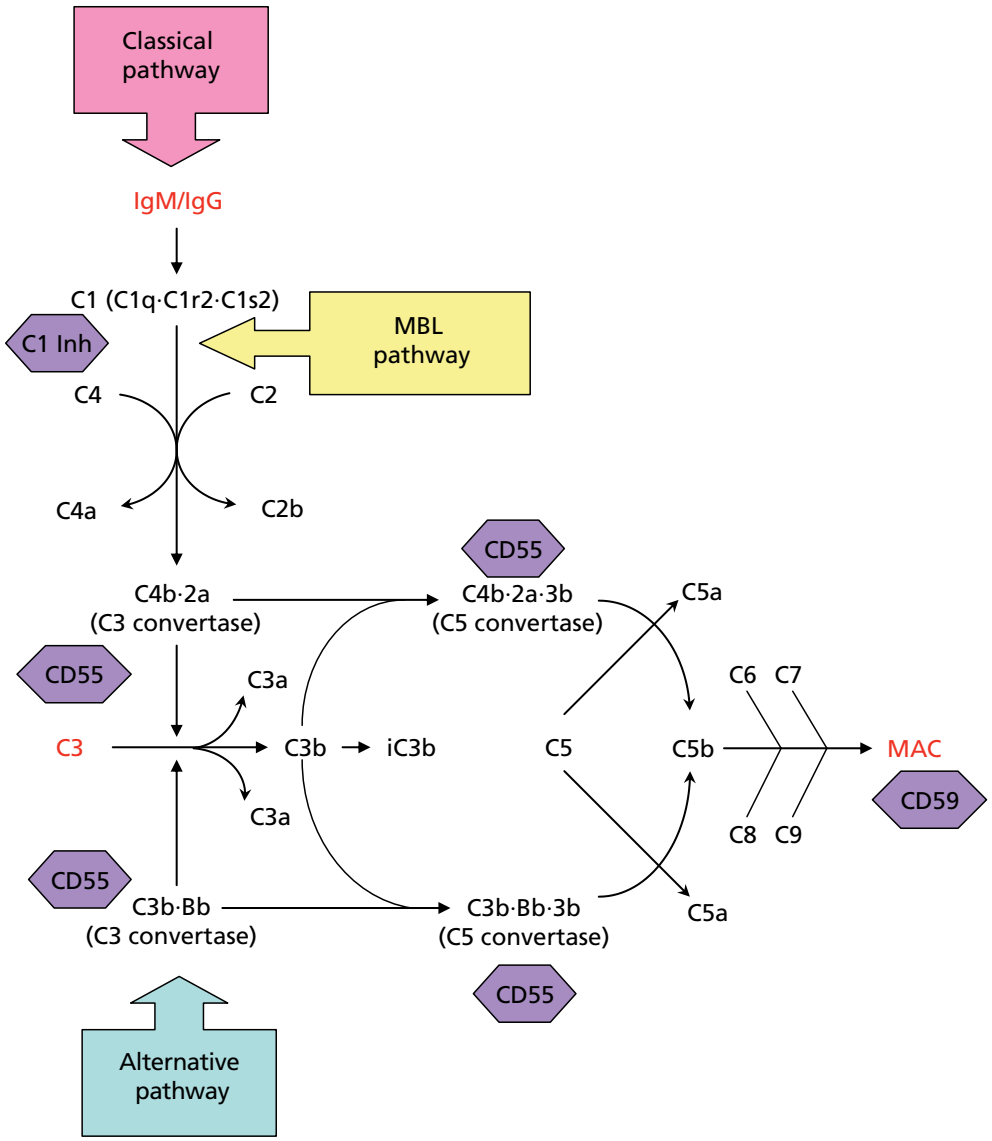

Figure 4.3 The complement cascade comprises three distinct pathways; the classical, mannose-binding lectin (MBL) and alternative pathways. The classical pathway is initiated by the binding of C1q, a component of C1 complex, to the CH2 region of the Fc domain of an antigen-bound antibody. Subsequently, C1s cleaves C4, then C2, resulting in the formation of C3 convertase. The MBL pathway is initiated when host MBL binds to mannose and fucose residues on a suitable surface such as a pathogen. The alternative pathway is constitutively activated at a low rate and is amplified on C3 cleavage. All three pathways converge at C3. C3 is cleaved by C3 convertase into C3a and C3b. C3b is an acceptor site for C5, which initiates formation of MAC. The complement cascade can be inhibited at various points by the C1 esterase inhibitor (C1 Inh), CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis).

the membrane attack complex which forms a pore in the membrane of the target cell, bringing about their lysis.

Two other pathways exist, the *alternative* and the *mannose-binding lectin pathways*, which do not necessarily involve antibody and represents non-specific 'innate' immunity. The alternative pathway is constitutively activated at a low rate and is amplified on C3 cleavage. The mannose-binding lectin pathway is initiated when host mannose-binding lectin binds to mannose and fucose residues on pathogen surface. All three pathways converge at C3.

The complement cascade is closely regulated by a number of complement defence molecules. For example, C1 inhibitor is a soluble protein that regulates C1, whilst the membrane protein CD55 inactivates C3 and C5 convertases by binding to C3b and C4b and CD59 binds to C8 and C9 to prevent formation of the membrane attack complex.

Neutralising activity

Antibodies can also mediate effects without effector molecules and immune effector cells by binding to and blocking a receptor, or neutralisation. In infection, protective antibodies bind to an organism's receptor to interfere with its attachment to host tissues. For instance, in COVID-19, caused by the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus enters the host cell by attaching its transmembrane spike protein to the angiotensin-converting enzyme 2 (ACE2) receptor. Host antibodies that bind to the viral spike protein, in particular the receptor-binding domain within the spike protein, are able to prevent virus entry. Neutralisation can also occur through other means e.g. multimeric IgA and IgM antibodies are able to bind to and aggregate organisms to inhibit their function and reduce infectivity.

The B cell receptor

The membrane-bound antibody together with Igα (CD79a) and Igβ (CD79b), forms the B cell receptor (BCR), which transmits a low-level basal signal critical for B-cell survival. (Figure 4.4) Upon engaging antigen, BCR aggregation is triggered, leading to phosphorylation of Immunoreceptor Tyrosine-based Activation Motifs (ITAM) within the cytoplasmic tail of CD79a and CD79b. The phosphorylated ITAMs then recruit and activate Syk kinase, which in turn recruit adaptor proteins and kinases such as Bruton's tyrosine kinase (BTK). This further leads to the activation of phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ2 (PLCγ), and mitogen-activated protein kinase (MAPK) pathways, and induction of NF-κB activation to modify B-cell survival, differentiation and proliferation.

As BCR signalling is critical to cell survival, these pathways can be targeted by drugs to treat B-cell malignancies, such as small molecular inhibitors against BTK, ibrutinib, in CLL and mantle cell lymphoma. Conversely, activation of the BCR signalling pathway has been observed in some subtypes of B-cell malignancies e.g. the activated B-cell like (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) as a result of gain-of-function mutations of CD79B and/or MYD88, with the latter mutation also implicated in lymphoplasmacytic lymphoma.

T cells

T-cell development

Although the earliest progenitors of T cells are produced in the bone marrow, the development and selection of the most mature immunologically competent T cells occur in the thymus. Despite the fact that the thymus is largest in neonates and gradually involutes with age, ceasing its activity after the fourth decade of life. The naive T cell pool can be maintained in adults through peripheral division and have a lifespan of 5–10 years.

Similar to B-cell development, the maturation of T cells is marked by rearrangement of *T cell receptor* (*TCR*) genes and expression of a number of key surface antigens. The *TCR* (Figure 4.5) also has certain similarities to immunoglobulin in that it is composed of two non-identical polypeptide chains that have constant and variable regions. There are two types of *TCR*, with αβ T cells expressing a heterodimer of an α-chain and a β-chain, and the minority population of γδ T cells expressing the γδ *TCR* heterodimer. A number of different molecules are associated with the two *TCR* polypeptide chains in order to generate the *TCR* complex. This transmembrane signalling complex of molecules is collectively known as CD3 (Figure 4.5) and is linked to second messenger signalling molecules, whose expression varies between different T-cell subsets and at different stages of T-cell differentiation.

The early T cell progenitors that arrive in the thymus do not express the *TCR* or the signature antigens of T cells (CD3, CD4 and CD8) and are termed *double negative* (CD4–CD8) cells. These cells are identified by expression of CD34 and CD7 and can be further subdivided based on expression of interleukin-2 receptor α chain (CD25) and CD44, an adhesion molecule. Interaction with the thymic stroma is accompanied by expression of CD2 and cytoplasmic expression of CD3 genes. By this stage, the cell is committed to the T-cell lineage and *TCR* gene rearrangement is underway.

Like *BCR* rearrangement, *TCR* β, γ, α and δ gene rearrangement is mediated by RAG-1 and RAG-2 enzymes. Rearrangement of the gene encoding the *TCR* β-chain occurs first with D to J rearrangements followed by V–D–J rearrangements to create a pre-*TCR*, which complexes with CD3 and upregulation of CD4 and CD8 to produce double positive cells. *TCR* α-chain gene rearrangement is then initiated to produce an αβ *TCR*.

During early fetal development, the first cells to leave the thymus as mature T cells have successfully rearranged their γ and δ genes and express the γδ form of the *TCR*. During later stages of human fetal development and throughout the rest of life, 85–98% of T cells that leave the thymus have undergone successful α and β gene rearrangement and express the αβ *TCR*.

Double-positive cells undergo selection to give rise to single-positive ($CD4^+$ or $CD8^+$) thymocytes which then migrate to the periphery as naive T cells ($CD45RA^+$ $CCR7^+$). There are two key thymic selection processes, *positive selection* and *negative selection*. Positive selection is the process whereby thymocytes that express receptors that are able to recognise peptides presented by major histocompatibility complex (MHC) molecules, can proceed through development. Cells that fail positive selection will die by neglect and apoptose within the thymus. In contrast, in negative selection, thymocytes that bind to self-expressing MHC molecules alone with high affinity, or self-antigen complexed with self-MHC, are eliminated. Both processes are critical for the generation of effective but tolerant T cells. It has been estimated that

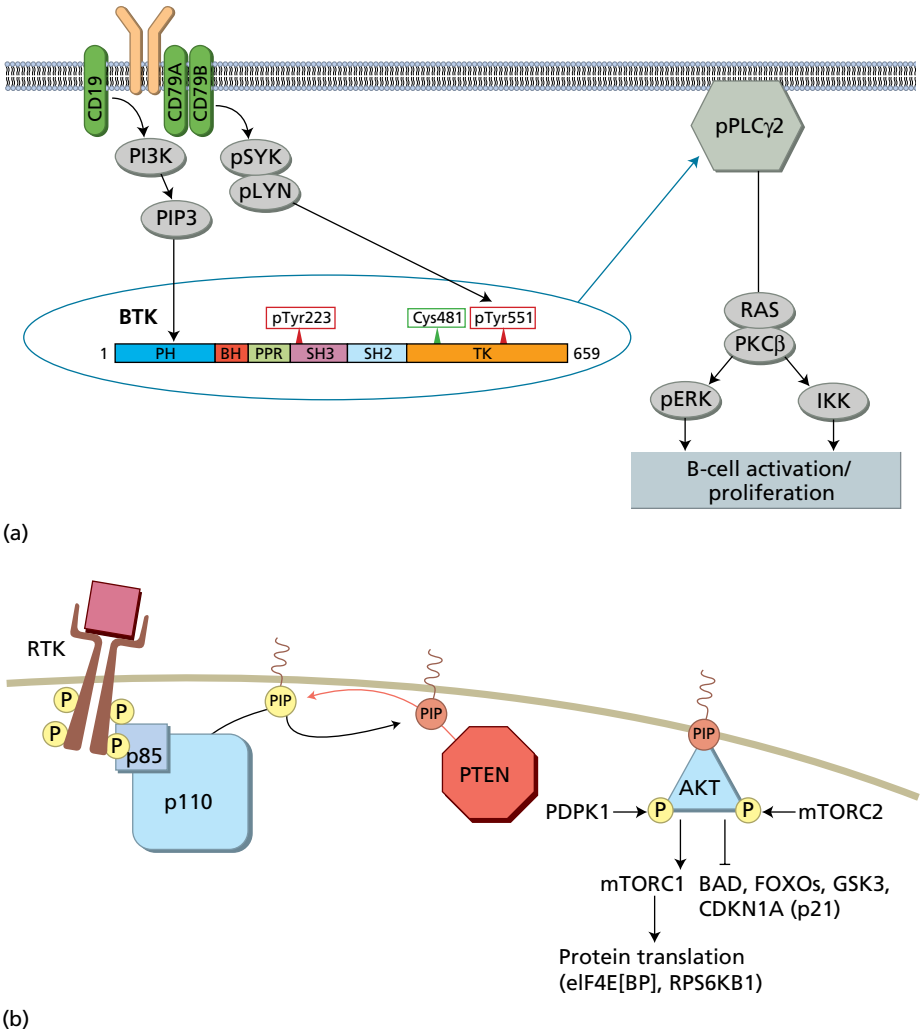

Figure 4.4 (a) BTK and its involvement in BCR signalling. Bruton's tyrosine kinase (BTK) comprises several domains including the: (i) pleckstrin homology (PH) domain, which targets BTK to the plasma membrane, (ii) BTK homology (BH) domain, (iii) polyproline (PPR) domain, (iv) Src homology (SH3 and SH2) domains (SH2 domain of BTK binds to the B cell adapter protein BLNK, which is required for full BTK activation) and (v) tyrosine kinase (TK) domain. Activation of BTK requires phosphorylation and binding of PIP3. Following *BCR* engagement and activation, and following PIP3 binding, BTK translocates to the plasma membrane, where it is phosphorylated at Tyr551 by LYN and SYK, leading to autophosphorylation of Tyr223. Once bound to BLNK, BTK phosphorylates PLCG2 at several sites, leading to calcium mobilisation and activation of the protein kinase C (PKC) family members and other effectors, including RAS, and to NFκB activation and phosphorylation of ERK. Most BTK inhibitors bind irreversibly to Cys481 within the ATP binding site of the BTK kinase domain, thus preventing BTK activation. Apart from its role in BCR signalling, BTK is also implicated in cytokine and Toll receptor signalling pathways. (Source: Hutchinson and Dyer (2014) *Br. J. Haematol.* **166**: 12–22. Reproduced with permission of John Wiley & Sons.) (b) A schematic representation of the phosphoinositol-3 kinase pathways. Extracellular ligands bind receptor tyrosine kinases which are subsequently (auto) phosphorylated. The p85 regulatory subunit of PI3K then binds to one of these phosphates and activates the p110 catalytic subunit. PI3K takes a phosphate from a donor ATP and adds to an acceptor phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3). This action is opposed by PTEN, which dephosphorylates PIP3. PIP3 can recruit activated (phosphorylated) AKT to the membrane; once PIP3 is bound to the PH domain of activated AKT a variety of downstream substrates are phosphorylated. In the B cell, these targets include BTK. (Source: Blachly and Baiocchi (2014). Reproduced with permission of John Wiley & Sons.)

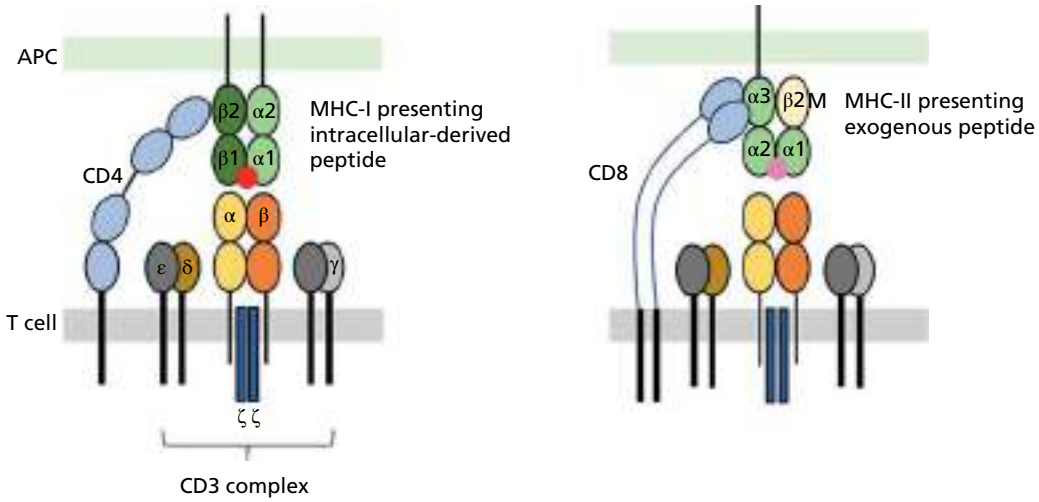

Figure 4.5 T cells recognise peptides held in MHC molecules. A class I MHC molecule is depicted on the left and a class II MHC molecule on the right. Some of the protein produced within each cell is broken down into peptides, which are presented on the cell surface in the peptide-binding groove of MHC molecules, usually class I. Extracellular molecules are taken up by antigen-presenting cells, broken down within the cell and presented on the cell surface in the peptide-binding groove of MHC molecules, usually class II. There are three isotypes of class II molecules, known as DP, DQ and DR, and three main class I isotypes, A, B and C. All of these are encoded within the *MHC* gene complex at 6p21.3. The genes encoding the peptide-binding grooves of each of these isotypes show extraordinary variability within the human population, so-called 'allelic polymorphism'. The range of peptides that can be held by different MHC molecules varies. Consequently, this polymorphism is reflected in differences between individuals in the ability to recognise specific peptides. Any one T-cell receptor (TCR) will only recognise a particular peptide within a particular groove structure. β_2-Microglobulin is a non-polymorphic immunoglobulin-like domain that is non-covalently associated with HLA class I MHC molecules; it stabilises peptide binding and is essential for the expression of class I on the cell surface. The αβ T-cell receptor (TCR) complex. The αβ TCR is composed of two polypeptide chains, each with a variable (open ovoid) and constant (closed ovoid) domain. Peptide plus MHC is recognised by the combined variable regions. The TCR is surrounded by the CD3 complex of transmembrane signalling molecules. This is composed of four types of polypeptide chain, γ, δ, ε and ζ. Most peripheral T cells express CD4 *or* CD8 with their TCR.

95% of all thymocytes die within the thymus either as a result of failure to rearrange their TCR genes in an expressible form or as a consequence of elimination during the T-cell selection process.

Thymic epithelial cells in the cortex are critical in the positive selection of thymocytes that have moderate affinity for MHC–peptide complexes. The boundary of the cortex and medulla is populated by macrophages (often called sentinel macrophages) that phagocytose cells undergoing apoptosis. The medulla contains medullary thymic epithelial cells (mTEC) which mediate the process of negative selection. Interestingly, these cells express the autoimmune regulator (Aire) protein which leads to transcriptional expression of a wide range of cellular genes, therefore ensuring that tolerance is achieved to proteins expressed in a range of peripheral organs. Structures known as Hassall corpuscles, which are whorled aggregates of epithelial cells, can also be seen in the medulla though their function is uncertain.

Antigen recognition by the TCR

T cells recognise peptides presented in association with MHC molecules. In humans, the MHC molecules are also known as human leucocyte antigens (HLA) and there are two classes of MHC molecules. MHC class I molecules are expressed by all nucleated cells except germ cells. They are not expressed by erythrocytes but are found on the surface of erythroblasts. The peptides recognised by T cells in association with class I MHC molecules are, in most circumstances, derived from proteins produced within the cell. This places MHC class I molecules in an excellent position to present peptides derived from viral proteins following intracellular viral infection (Figure 4.6). It is now clear that antigen-presenting cells are also able to take up proteins from outside the cell and process them such that peptides gain access to the MHC class I presentation. This process is called *cross-presentation* and

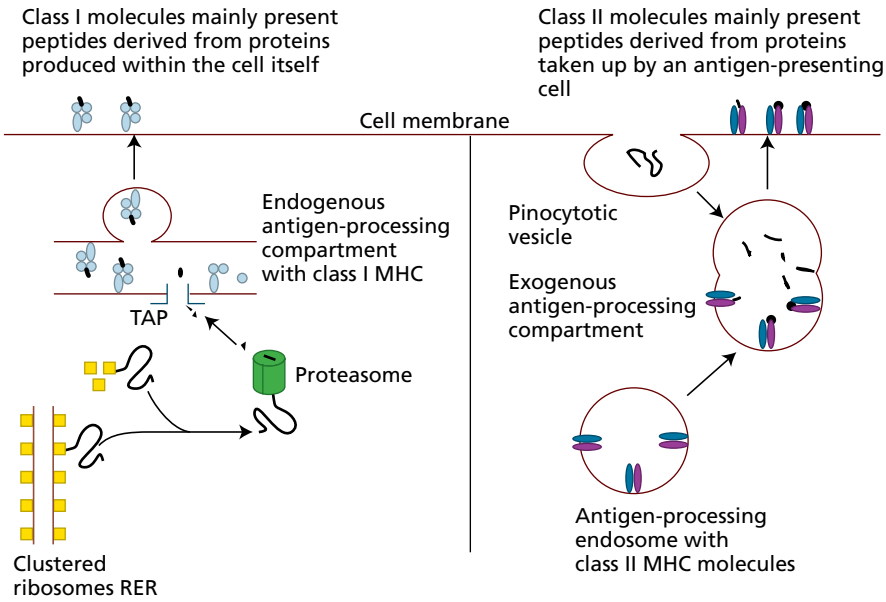

Figure 4.6 Antigen processing and presentation in association with MHC molecules. (Left): Antigen presentation of proteins produced within cells is mainly the property of class I MHC molecules. A proportion of proteins (black ribbons) produced within a cell on ribosomes (yellow) are broken down to peptides (black fragments) within a cytoplasmic molecular complex known as a proteasome. The resulting peptides are actively transported by TAP proteins (blue) through the wall of specialised endoplasmic reticulum (ER) that has MHC class I molecules in its wall. The peptides and β_2-microglobulin associate with class I molecules and are then expressed on the cell surface. (Right): Antigen presentation in association with class II MHC molecules involves pinocytosis of antigen (black ribbon) and fusion with an antigen-processing endosome, which has class II MHC molecules (magenta and blue) inserted in its wall. The antigen-presenting grooves of the class II MHC molecules are kept empty by the association with invariant chain (CD74). Fusion of the pinosome with the endosome heralds the activation of proteolytic enzymes; the invariant chain and the ingested proteins are broken down to peptides. The resulting peptides (black fragments) are assembled into the antigen-presenting groove of class II MHC molecules that are held in the endosomal wall. The HLA class II molecules with bound peptides are then carried to the cell surface.

may be particularly important in generating $CD8^+$ T cell responses to tumour-associated proteins.

MHC class I antigen presentation starts with the intracellular breakdown of proteins by a multimolecular proteolytic complex known as a *proteasome*. These peptides are actively transported by TAP (transporter associated with antigen processing) proteins into the endoplasmic reticulum, where empty MHC class I molecules are being assembled. The nascent MHC molecules are able to 'fold' around the peptides, which make non-covalent interactions with the peptide-binding groove at the top of the molecule. This complex is then stabilised by the association of β_2-microglobulin before being transported to the cell surface. In this way, the cells are continuously advertising the peptide composition of the proteins that they are producing.

T cells that recognise peptide presented by MHC class I molecules express a molecule, CD8, that binds to the α3 domain of the MHC class I molecule. When any cell in the body presents immunogenic peptides, these may be recognised by a cytotoxic CD8 T lymphocyte that can then kill the target cell. This is most likely to occur as the result of virus infection when virus-encoded proteins are produced; it may also occur following the acquisition of a genetic mutation within a cell.

The other class of MHC molecule, MHC class II, is less widely expressed. The only cells that constitutively express large amounts of this class of MHC molecule are specialised antigen-presenting cells e.g. dendritic cells, B lymphocytes and thymic epithelial cells. Dendritic cells share a pattern of haematopoietic differentiation with monocytes and in the laboratory, they can be derived from blood mononuclear preparations by culture with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. They migrate to many tissues, particularly epithelia, where they remain until activated by local tissue injury. On activation, they take up fluid and particles from their surrounding environment and digest proteins, which they present as MHC class II–peptide complexes at the cell surface.

The scrutiny of antigen-presenting cells by T cells starts when dendritic cells move from peripheral tissues to the T-cell-rich areas of adjacent secondary lymphoid organs. In this site, they are known as interdigitating dendritic cells (IDC), which express

molecules associated with T-cell activation such as CD40, CD80 and CD86. In the T zones of secondary lymphoid organs, recirculating T cells are constantly migrating to the surface of IDC, which they scrutinise for the presence of an MHC–peptide complex to which they can bind with their TCR. In this way, the T cells continually screen for the presence of peptides derived from both extracellular and intracellular antigens.

CD4⁺ and CD8⁺ T cells and their functions

$CD4^+$ T cells are multifunctional. Their major role is in the provision of signals that induce proliferation or differentiation of T or B cells, generally known as *T-cell help*. They can also activate macrophages and may mediate HLA class-II-restricted cytotoxic activity. Increasing attention is being given to their regulatory role in T-cell immune responses and this is mediated through a Forkhead transcription factor (FoxP3)-positive subset of T regulatory cells.

$CD8^+$ T cells have a predominant role in the recognition of virally infected cells and recognise antigen in association with HLA class I. Antigen recognition is followed by lysis of the target cell and secretion of cytokines such as interferon (IFN)-γ. There are two main killing mechanisms, which both induce apoptosis in the target cell. Firstly, enzymes known as *granzymes* and pore-forming molecules termed *perforins* are transferred from the T cell to the target cell. Granzymes and perforins are present in granules that stain crimson and are easily seen against the featureless pale cytoplasm of cytotoxic T cells and NK cells in Jenner–Giemsa preparations. These features have given rise to the term *large granular lymphocyte*. The second cytotoxic mechanism involves the expression of the Fas ligand (FasL, CD95L) by the effector cell. The resulting engagement of Fas (CD95) on the target cell induces the target to undergo apoptosis. Fas is a member of the TNF receptor family and FasL is an analogue of TNF-α and TNF-β.

Differentiation of primed T cells into effector cells

Following the first recognition of antigen or *T-cell priming*, naive $CD8^+$ T cells enter an *expansion phase* whereby they differentiate and proliferate into effector $CD8^+$ T cells which then clear the infection or antigen-expressing cells. This is followed by a *contraction phase* whereupon most of the effector $CD8^+$ T cells apoptose to leave behind a *memory phase*. During this phase, the cells differentiate into memory cells which can react rapidly to reinfection. Memory cells can be subdivided into *central memory*, *effector memory* or *stem cell memory* cells. The central and effector memory cells both produce effector cytokines but only the central memory cells have high proliferative capacity. Stem central memory cells are rare and have no effector function but have high self-renewal and proliferative capacity. After the contraction phase, small numbers of terminal effector $CD8^+$ T cells ($CD45RA^+CCR7^-$) can persist especially in the context of a chronic and persistent insult. These cells have low proliferative capacity but produce high levels of interferon γ (IFNγ).

$CD4^+$ T cells are critical for the development of an effective host immune response. The consequences of low numbers of $CD4^+$ T cells are clearly demonstrated in HIV-infected individuals, who develop malignancies and opportunistic infections. Differentiation of primed $CD4^+$ cells is directed into one of five major pathways depending on the nature of the antigen, associated inflammatory stimuli and the affinity of T-cell engagement. In general, $CD4^+$ cells are highly plastic and can switch from one phenotype to another. For instance, $CD4^+$ T cells stimulated in the presence of IL-12, which is produced by macrophages and dendritic cells, are more likely to differentiate into Th1 cells. But if subsequently stimulated with IL-10, it can change into a regulatory phenotype, especially in chronic inflammation.

Similar to $CD8^+$ T cells, $CD4^+$ T cells undergo expansion, contraction and memory phases to generate effector and memory subsets. In addition, they can be subdivided into five major $CD4^+$ T cell subsets:

1. *Th1 cells*
   As above, release of IL-12 by macrophages and dendritic cells activate the transcription factor Tbet, in naive $CD4^+$ T cells to drive IFNγ release, and differentiation of the cell into a Th1 phenotype. This inflammatory subset is important for the clearance of intracellular pathogens such as viruses and bacteria. The cells are cytolytic and can kill infected macrophages through granzyme/perforin or Fas ligand. This subset is also conventionally regarded as characteristic indicative of an anti-tumour response.
2. *Th2 cells*
   IL-4, produced by activated Th2 cells itself, drives the transcription factors GATA3 and STAT5 to induce Th2 differentiation. Th2 cells produce cytokines associated with antibody production, particularly IL-4, IL-10 and, in some instances, IL-6, although the last of these is mainly produced by macrophages and osteoclasts. The balance between Th1 and Th2 cells can be an important determinant of immunopathology. Tuberculoid leprosy exemplifies apparent dominance of a Th1 response, while atopic disease seems to reflect an imbalance towards Th2. Nevertheless, manipulation of these cytokine networks provides a potential means of modifying established immune responses to avoid the complications associated with over activity of either Th1 or Th2 cells.
3. *Th17 cells*
   The immunosuppressive cytokines TGF-β, IL-6 and/or IL-23, activates the transcription factor retinoic-acid receptor-related orphan receptor γt (RORγt), which is a master regulator for Th17 differentiation. As implied by its name, these cells produce large amounts of the cytokine IL-17, a pro-inflammatory cytokine. These cells play an important role in

the control of fungal disease and defence against extracellular pathogens, but are also implicated in autoimmune disease.

4 *TFH cells*

Follicular helper T cells (TFH) are crucial in providing B-cell help. Differentiation of naive CD4⁺ T cells in TFH is driven by BCL-6, which is also vital in promoting B-cell proliferation in the germinal centre and suppression of B-cell differentiation. TFH cells are attracted into the follicles via CXCR5-CXCL13 interaction. The co-localisation of Tfh and B cells provides CD40L-CD40 costimulation, required for germinal centre maintenance. The inducible costimulatory molecule (ICOS) upregulated on TFH cells, is also necessary for maintenance of CD40L expression on TFH cells.

Regulatory CD4⁺ T cells

Regulatory T (Treg) cells are most abundant in childhood where they keep the immune system in check during this period of recurrent, new antigen exposure. However, they also play an important role in the control of inflammation, allergy and tissue repair and regeneration. *Foxp3* is the master regulator of Treg cells. Mutation or deficiency of Foxp3 in both human and mice are associated with profound and lethal autoimmunity. Phenotypically, these cells express CD4, CD25 (IL-2 receptor α-chain) and low levels of CD127 (a component of the IL-17 receptor).

Broadly, there are two types of Treg cells, *thymic* or *central Treg* cells, which comprise the majority of Treg cells, and *peripheral* or *inducible Treg* cells. As discussed in the section on *T-cell Development*, maturing thymic T cells that react with self-MHC/peptide are deleted by negative selection. Inevitably, some self-reactive T cells will escape and these will be diverted into Treg differentiation. The exact developmental pathway of these cells is likely to depend on the signal strength between the TCR and presented self-peptide as well as availability of co-stimulatory signals.

In the periphery, mature CD4⁺ T cells are polarised to Treg cells by TGF-β, a critical cytokine required for Foxp3 induction. The subsequent survival and proliferation of these cells relies on IL-2, itself produced by activated CD4⁺ T cells, thus producing a negative feedback loop to control T-cell stimulation.

There are multiple ways in which Treg cells can suppress CD4⁺ and CD8⁺ T cells activation, proliferation and function, and activation of antigen-presenting cells. These include secretion of immunosuppressive cytokines (TGF-β and IL-10), compete for growth factors and engagement of MHC class II molecules, and cell–cell dependent suppression. As an example, upon antigen exposure, antigen-specific Treg cells are recruited to the site via chemokines, where they out-compete antigen-specific naive T cells. These Treg cells then downregulate dendritic cell function by directly reducing the expression of co-stimulatory molecules (CD80/CD86).

The role of co-stimulatory and inhibitory molecules

Whenever an immune effector cell recognises a potential antigen on a target cell, the decision as to whether or not the effector cell is activated to kill the target cell depends on the balance of activatory and inhibitory signals received by the immune effector cell. For B cells, this may be the balance between activatory and inhibitory FcγRIIb receptors. For T cells, the immunoglobulin superfamily and *tumour necrosis factor receptor superfamily* (*TNFRS*) are the two major classes that encode such molecules. Conventionally, T-cell activation is viewed in a stepwise manner. *Signal one* is triggered when a naive T cell recognises MHC-bound antigen on the surface of an antigen-presenting cell. CD4 or CD8 will bind MHC simultaneously to stabilise this interaction. To fully activate a naive T cell and initiate its differentiation and proliferation into effector cells, a second signal is required. *Signal two* can be provided by CD28 on T cells, which interacts with either CD80 or CD86 on the antigen-presenting cell to induce T-cell proliferation. To avoid uncontrolled cell proliferation, T-cell activation is accompanied by upregulation of the *cytotoxic T-lymphocyte-associated antigen 4* molecule (*CTLA-4*, CD152) on T cells, which is an inhibitory molecule that competes with CD28 for ligand binding.

Apart from CD28, which belongs the immunoglobulin superfamily, TNFRS members (e.g. OX40, 4-1BB, CD27) can replace CD28 as co-stimulatory molecules. Cytotoxic T cells are less dependent on CD28 but require signals from CD27 or 4-1BB (CD137). Similarly, there are a number of co-inhibitory molecules which contribute to control of T-cell activation such as programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3).

A *third signal* is important for the differentiation of T cells into the defined subsets described in *Differentiation of primed T cells into effector cells*, to enable them to carry out specific tasks. As an example, exposure IL-17 will skew the antigen-experienced, activated T cell into a Th17 phenotype for tissue repair.

Natural killer cells

Unlike T and B cells, NK cells are able to kill infected or malignant cells without prior sensitisation. NK cell-killing is induced when a 'stressed cell' downregulates receptors that are normally expressed on its surface. Under a normal state, these receptors prevent NK cell-killing by binding to inhibitory NK receptors. As an example, MHC class I molecules on nucleated cells bind to inhibitory killer-cell immunoglobulin-like receptors (KIR) on NK cells to keep them in an inhibitory state. Downregulation of class I molecules, e.g. in tumour cells, leads

to loss of NK cell-inhibitory signalling and subsequent killing of the target cell. This is known as '*missing self*' recognition. Similar to cytotoxic T cells, NK cells trigger cell death through release of cytotoxic granules (granzymes and perforin) and death receptor pathways (Fas ligand/Fas receptor). NK cells are also able to modulate T- and B-cell responses through release of cytokines (IFNγ, TNFα) and chemokines (CCL3, CCL4).

NK cells can also be activated through other pathways. Stressed cells often upregulate new molecules on their surface e.g. MHC class I polypeptide-related sequence A or B (MICA/B), which are ligands for activatory receptors on NK cell (e.g. natural-killer group 2, member D [NKG2D]). Activation of the NK cell triggers cytotoxicity.

NK cells are conventionally recognised as $CD3^-$ $CD56^+$ cells. They can be further divided into cells that express low ($CD56^{dim}$) or high levels ($CD56^{bright}$) of CD56. The $CD3^-$ $CD56^{dim}$ population form the majority of peripheral blood NK cells whereas the $CD3^-$ $CD56^{bright}$ cells primarily reside in tissues and are more cytotoxic. As discussed, NK cells express a range of inhibitory and activating receptors and it is the balance of signals received through these molecules that determines whether the target cell will be recognised. Specifically, inhibitory signals include: (i) killer inhibitory receptors (KIRs) that bind to HLA-C or HLA-B alleles on the surface of the target cell and (ii) CD94–NKG2 heterodimers that bind to HLA-E. Activating receptors include: (i) activating forms of KIR molecules, whose ligands are uncertain at present, (ii) NKG2D, which binds to proteins such as MICA and ULBP expressed on cells under physiological stress and (iii) the activatory FcγR, FcγRIII (CD16).

HLA class I expression is often downregulated on the surface of cells following viral infection and is also a common feature of malignant cells. This provides some protection from recognition by $CD8^+$ T cells but renders the cell susceptible to lysis by NK cells. Thus, $CD8^+$ T cells and NK cells can be viewed as having complementary recognition systems based on the level of HLA class I on the target cell.

Natural killer T cells

Natural killer T (NKT) cells represent a small (<1%) population of peripheral blood T cells and appear to be positioned somewhere between conventional T cells and NK cells in that they express a TCR, but also a range of receptors typically associated with NK cells. Moreover, the TCR is invariant, with the same Vβ11Vα24 heterodimer being expressed on all cells. NKT cells recognise glycolipid antigens presented on the surface of non-polymorphic, MHC class I-like CD1d molecules. NKT cells can be subdivided into type 1 or type 2 NKT cells. Type 1 NKT cells recognise a prototypic glycolipid, α-galactosylceramide (α-GalCer), thought to originate from microbes found on marine sponges. In contrast, type 2 NKT cells have a broader lipid antigen profile and do not recognise α-GalCer.

NKT cells are similar to NK cells in that they are activated very early in an immune response. *In vitro* studies show that these cells can be polarised to produced cytotoxic or immunoregulatory cytokines, and contribute to anti-microbial and anti-tumour immunity.

The immune response in cancer and its therapy

The concept that the immune system is able to participate in the control and growth of cancer or '*immunosurveillance*', was suggested by Burnet and Thomas in 1957. However, it was not until the 1990s that incontrovertible evidence of immunosurveillance was demonstrated. A crucial piece of evidence arose with the generation of RAG-2 mice which lack T, B and NKT cells. Without a functioning adaptive immune system, these mice were more susceptible to development of carcinogen-induced sarcomas and epithelial tumours compared to animals with normal immune system. Immunosurveillance can be described in three different stages. The first is the *elimination* stage, where the immune system is ability to kill the tumour. However, if tumour cells survive this phase they enter into an *equilibrium* with the host, where the immune system is able to control but not fully eliminate the tumour. During this stage, the immune system inadvertently selects for resistant tumours capable of surviving continued immune attack. Finally, during the *escape* phase, the evolved tumour overcomes host immunity and grows in an uncontrolled tumour. As cancer arises as a result of failure of the host immune system, cancer immunotherapy rationalises that the immune system can be 'reawakened' to control the tumour.

Allogeneic stem cell transplantation, which relies on the anti-tumour effect from donor lymphocytes is the oldest form of immunotherapy and is discussed in more detail in Chapter 37. However, cancer immunotherapy did not really come of age until 1997, when the CD20 monoclonal antibody, rituximab, was approved for use in treatment of B-cell lymphoma. Rituximab provided the proof-of-concept that monoclonal antibodies can be used to reawaken the immune system. Here, rituximab binds to CD20 via its Fab domains and macrophages via Fc-FcγR interaction to kill lymphoma cells through ADCP (*Fc–Fc receptor-mediated interactions*). NK cell-mediated ADCC and complement-dependent killing have also been proposed, but as none of these mechanisms directly engage normal B or T cells, a memory response is rarely observed, and relapses occur in a significant proportion of patients. This in part, prompted researchers to focus on monoclonal antibodies which are T-cell antigens, in particular, those binding to co-stimulatory or co-inhibitory molecules (*The role of co-stimulatory and inhibitory molecules*). Of these, monoclonal antibodies targeting the T-cell co-inhibitory pathway or, PD-1/PD-L1 (*checkpoint inhibitors*)

have shown the most clinical efficacy. These antibodies either bind to 'exhausted' PD-1 expressing T cells or PD-L1 on macrophages or tumour, in the case of Hodgkin and primary mediastinal B-cell lymphoma, to disrupt negative feedback signalling into tumour-specific cytotoxic T cells, thereby allowing them to kill the tumour cells (see Chapter 6 for more detail). However, akin to CD20 monoclonal antibodies, the effects of PD-1 blockade are thought to be relatively short-lived as the exhausted T cells are transcriptionally hardwired into a distinct exhausted lineage and do not acquire a memory phenotype with treatment.

Significant advances in the TCR, its co-stimulatory receptors and genetic engineering have led to the development of artificial *chimeric antigen receptors* (CAR). This involves grafting the genetic sequence of antigen-binding domains of a monoclonal antibody (e.g. CD19) onto intracellular signalling domains (e.g. ζ chain of TCR molecule and 4-1BB signalling domains), and then transfecting this gene into lymphocytes (commonly T or NK cells) using a viral or non-viral vector. This redirects the specificity of the lymphocyte such that it is now able to kill target cells that express the target for the monoclonal antibody. The greatest success so far has been in the use of CD19-targeted CAR which is used in the treatment of B cell malignancy (see Chapter 6).

With the same purpose of redirecting T-cell specificity, bispecific antibodies can perform the same function. These are essentially monoclonal antibodies engineered to bind to two different targets e.g. CD20 on B cells and CD3 on T cells. In doing so, the bispecific antibodies direct T-cell cytotoxicity towards the CD20-expressing B cell.

Interpretation of blood lymphocyte counts

Blood provides the most accessible view of the lymphoid system, but it must be remembered that peripheral blood contains only around 2% of total body lymphocytes. The cells present in the blood are in transit and many are recirculating T and B lymphocytes that will pass rapidly into secondary lymphoid organs in less than 30 minutes.

The numbers of different lymphocyte subsets normally found in the blood of different age groups are given in Table 4.1. These numbers are derived from studies in which whole blood was labelled by fluorescent dye/monoclonal antibody conjugates, followed by red cell lysis and flow cytometry. When this method is used to measure the proportion of lymphocytes that belong to different subsets, much of the inter-laboratory variation that is observed in calculating absolute numbers of subsets can be attributed to the method of measuring the total white cell count and the percentage of lymphocytes. A wide range of results is to be expected between different individuals. The most consistent variation is seen in childhood, but from adolescence onwards age-related changes are small and there is also little difference in relation to ethnicity or gender.

Alteration of the lymphocyte count can result from an absolute change in the number of cells or alteration in the distribution of lymphocytes within tissues. Redistribution of lymphocytes accounts for much of the variation in lymphocyte subset numbers found in serial measurements within a healthy individual. Some of these changes in lymphocyte number follow a diurnal pattern, with peak levels at night and nadir in the morning; accordingly, time of sampling should be taken into account.

Table 4.1 Normal ranges for lymphocyte subsets in the blood.*

	0–2 months		2–3 months		4–8 months		1–2 years		2–5 years		5–12 years		Adults	
Percentile	*5*	*95*	*5*	*95*	*5*	*95*	*5*	*95*	*5*	*95*	*5*	*95*	*5*	*95*
Total lymphocytes	3.2	8.5	2.9	8.8	3.6	8.8	2.2	8.3	2.4	5.8	1.8	5.8	1.0	3.4
$CD3^+$ (all αβ and γδ T cells)			2.1	6.5	2.3	6.5	1.5	5.4	1.6	4.2	0.9	2.6	0.6	2.5
$CD4^+$ (class II MHC-restricted αβ T cells)	1.2	5.3	1.4	5.6	1.4	5.7	1.0	3.6	0.9	2.9	0.5	1.4	0.35	1.5
$CD8^+$ (class I MHC-restricted αβ T cells, some γδ T cells and NK cells)			0.7	2.5	0.7	2.5	0.6	2.2	0.6	1.9	0.4	1.2	0.23	1.1
CD4/CD8 ratio	1.1	4.5	1.1	4.4	1.1	4.2	1.0	3.0	0.9	2.7			0.66	3.5
$CD3^-$ $CD57^+$ or $CD56^+$ NK cells					0.3	0.7			0.2	0.6			0.2	0.7
$CD19^+$ or $s\kappa^+$ or $s\lambda^+$ total B cells					0.5	1.5			0.5	1.3			0.04	0.7

There is considerable variation in the normal ranges reported from different studies and this table is only intended to be illustrative.
* All values (except CD4/CD8 ratio) are $\times 10^9$/L.

Increased number of effector cells in the blood usually reflects an active immune response. Analysis of the phenotype of these effector cells provides some information on the type of immune response, especially in differentiating between cytotoxic and inflammatory $CD4^+$ T cells. Particularly in viral infections, this response may be of sufficient magnitude to cause a lymphocytosis. Some non-malignant causes of lymphocytosis are given in Table 4.2.

Redistribution of lymphocytes is probably the cause of the lymphocytosis seen in *Bordetella pertussis* infection. Although lymphocytosis is uncommon in bacterial infections, in children over the age of 6 months the second and third weeks of infection with pertussis are usually associated with a lymphocytosis in excess of 10×10^9/L (in some cases $>50 \times 10^9$/L). The lymphocytosis consists of small lymphocytes and is believed to be caused by a protein toxin from *B. pertussis* that prevents migration of lymphocytes across endothelium into tissue.

Acute infectious lymphocytosis is a benign disease, usually in children. In most cases, there are no symptoms, but in some there is fever and in a small proportion gastrointestinal symptoms. Increase in the size of secondary lymphoid organs, anaemia and thrombocytopenia are rare. There is an increased number of small lymphocytes, persisting for 3–7 weeks, with an average peak level of $30–40 \times 10^9$/L. This is usually associated with an eosinophilia (average 2×10^9/L), but the aetiology of the condition is unknown.

Some *lymphopenias* predominantly reflect redistribution rather than a depletion of total body lymphocyte numbers. A dramatic short-term lymphopenia is induced by corticosteroids. This causes the retention of lymphocytes in secondary lymphoid organs but these are released again after about 2 days and the blood lymphocyte count returns to near-normal levels. Endogenous secretion of corticosteroids during acute illnesses may be partly responsible for the lymphopenias often seen in conditions such as heart failure or pneumonia. In many other conditions, lymphopenia reflects an increased rate of death of lymphocytes and/or a reduction in their rate of formation. A normal absolute lymphocyte count can belie an underlying deficit of one or more lymphocyte subsets. This is often seen in HIV infection, when a severe deficit of $CD4^+$ T cells may be disguised by expansion of $CD8^+$ T cells.

Table 4.2 Non-malignant causes of lymphocytosis.

Viral infections
Infectious lymphocytosis, infectious mononucleosis, cytomegalovirus infection; occasionally rubella, hepatitis, adenoviruses, varicella, HIV, human herpesvirus 6, mumps, chickenpox, dengue
Bacterial infections
Pertussis; occasionally healing tuberculosis, brucellosis, secondary and congenital syphilis, cat scratch fever, typhoid fever, diphtheria
Protozoal infections
Toxoplasmosis; occasionally malaria
Other conditions
Serum sickness, allergic drug reactions, splenectomy, dermatitis herpetiformis, metastatic melanoma, hyperthyroidism, congenital adrenal hyperplasia

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CHAPTER 5

The spleen

5

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The spleen is the largest secondary lymphoid organ, with diverse immunological functions in addition to its homeostatic role in the clearance of red blood cells. Splenic function and splenic tissue architecture are intimately related. Symptoms associated with splenic disorders may dominate the clinical picture, for example abdominal discomfort due to gross enlargement. This chapter will therefore review the basic structure and function of the spleen with emphasis on clinical considerations, before considering the spectrum of splenic disorders.

Structure and function of the spleen

The spleen lies in the left hypochondrium, with its long axis beneath the proximal half of the tenth rib. Its convex surface rests under the diaphragm, whereas the visceral surface is in contact with the stomach and left kidney, with the tail of the pancreas reaching the hilum on the medial side. The normal spleen weighs about 150–250 g, but there is considerable variation between normal individuals and at various ages in the same individual. At puberty, it weighs about 200–300 g but after the age of 65 years, this decreases to 100–150 g or less. In adults, its length is 8–13 cm, width 4.5–7.0 cm, surface area 45–80 cm^2 and volume less than 275 cm^3. Of note, up to 10% of people have accessory spleens, normally as a single piece of tissue that can be found in a variety of sites, either locally or more distantly in the abdomen. A spleen greater than 14 cm in cranio-caudal length is usually palpable (see below). It enlarges in a wide range of diseases and has been measured at more than 2 kg in some blood disorders.

The major functions of the spleen are: (i) filtration and quality control of red cells within the circulation, (ii) capture and destruction of blood-borne pathogens and (iii) generation of adaptive immune responses. In order to achieve these functions, the spleen has evolved a unique anatomical structure in which blood is filtered through two main systems. These consist of the white pulp (WP), concerned mainly with adaptive immunological function, and a red pulp (RP) which regulates the selection of red cells for re-entry into the circulation but also serves distinct immunological functions, particularly those of innate immune effector cells such as neutrophils, dendritic cells (DCs), monocytes/macrophages and gamma delta (γδ) T-cells. A major feature is the presence of both 'open' and 'closed' circulatory systems (see below).

The spleen is enclosed by a connective tissue framework that extends inwards to form a fibrous network. Blood enters the pelvis and the majority of terminal arterioles release their contents into an open network without traditional endothelial linings within the RP, before reentering the closed venous system (see below). There is no afferent lymphatic to the spleen and the efferent lymphatic system leaves along the route of the splenic vein. The spleen contains a large amount of lymphoid tissue that is mostly concentrated in concentric rings around the arterioles of the WP. This WP does not contain a capsule to separate it from the RP but instead there is a cellular border (perifollicular zone) largely comprising innate immune cells.

Splenic blood flow and the red pulp

The circulation within the spleen is illustrated in Figure 5.1. Blood is carried to the spleen via the splenic artery, which branches into the trabecular arteries and then arborises in a pattern that lacks

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

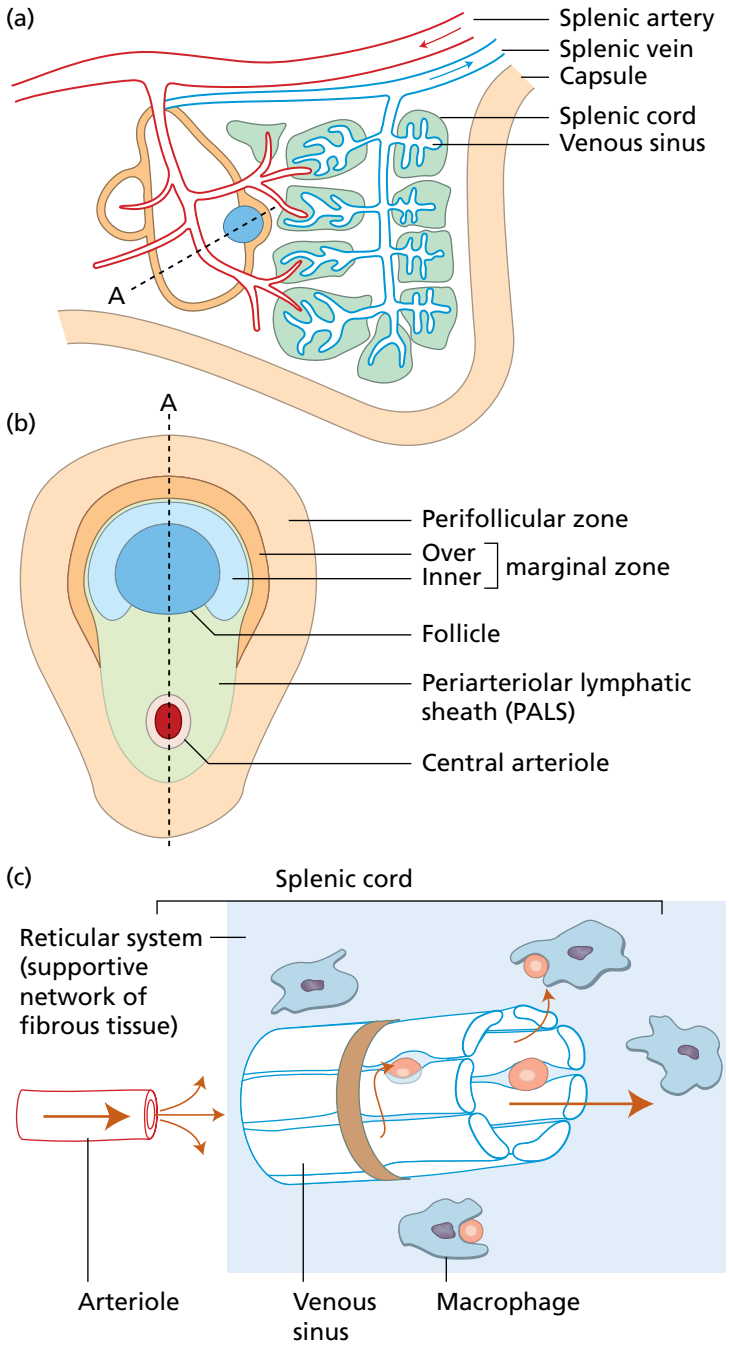

Figure 5.1 The vascular and lymphatic organisation of the spleen. (a) Schematic representation of blood flow in the spleen. Blood enters through the splenic artery and then breaks up into splenic arterioles. The white pulp is an aggregation of lymphoid tissue around these vessels. The arterioles then open into splenic cords lined with macrophages. Blood must then reenter the circulation by passing into a venous sinus. (b) Cross-section of white pulp at section A in (a). The central arteriole is surrounded by a periarteriolar lymphoid sheath of T-lymphocytes and occasional follicles that are rich in B-cells. The marginal zone is divided into an inner and outer zone around which there is a large perifollicular zone. (c) Schematic representation of the organisation of the red pulp. Blood cells are released by a splenic arteriole into a reticulum-lined splenic cord. Red cells that have flexible membranes are able to enter the venous sinus by passing through gaps in the endothelial lining. Cells that are unable to achieve this are 'filtered' from the blood through ingestion by macrophages (Source: Mebius and Kraal (2005). Reproduced with permission of Nature Publishing.)

interarterial connections and thus effectively generates end arteries. The terminal arteries acquire a coaxial sheath of lymphocytes containing lymphoid follicles and this together constitutes the WP (see below). These terminal arteries then split into many arterioles and capillaries, some of which terminate in the WP (perifollicular zone), while others go on to enter the RP.

In the RP, there are two major forms of blood circulation: a closed system, typical of the rest of the vascular system, in which arteries and veins communicate through endothelial-lined vessels, and an open circulation, in which arterioles terminate in free endings on splenic cords (also known as the cords of Billroth) and from which cells must subsequently cross a sinusoidal endothelial layer to reenter the circulation. The cords consist of a fibroblast-like reticular meshwork containing numerous macrophages and monocytes and they are critical to the filtration function of the spleen. Red cells transiting the cords must gain entry to a venous sinus if they are to reenter the systemic circulation. The sinuses are lined by endothelial and adventitial cells with a basement membrane and possess narrow interendothelial spaces through which flexible red cells

may pass. The passage of cells into the sinuses is controlled by their ability to squeeze through the interendothelial spaces, assisted by contraction of the reticular cells. Red cells with inflexible membranes, mostly elderly, are not able to pass through these gaps and are ingested by macrophages in the cords. The great majority of blood flows in this unique open system and only a minority of arteries connect directly to the trabecular vein in a typical closed vascular system.

There is thus both a rapid and a slow transit component in the splenic circulation. The rapid transit (closed circulation) is of the order of 1–2 min, whereas the slow transit (open circulation) has a circulation time of 30–60 min or even longer. In normal subjects, the blood flows through the spleen at a rate of about 5% of blood volume per minute, so that each day the blood has repeated passages through the spleen. During the flow, by a process called plasma skimming, the plasma and leukocytes pass preferentially to the white pulp, while the red cells remain in the axial stream of the central artery.

Blood pooling within the spleen

The normal red cell content of the spleen is 30–70 mL, which represents less than 5% of the total red cell mass. When the spleen is enlarged, there is expansion of the vascular bed. This results in a considerable pool with a high haematocrit and only a slow exchange of red cells with the general circulation. In states of massive hypersplenism, such as myelofibrosis, as much as 40% of the red cell mass may be pooled in the spleen. This pooling will functionally exclude a relatively large volume of red cells from the main arteriovenous circulation, and thus be an important cause of anaemia.

The normal spleen contains a reservoir of granulocytes that is in dynamic equilibrium with the circulating population. It represents 30–50% of the total marginating pool, with a mean transit time of about 10 min. Splenic sequestration of granulocytes is thought to be responsible for the neutropenia that often occurs in patients with splenomegaly. Platelets have also been shown to be significantly reservoired in the spleen and are rapidly interchangeable with the circulation. In normal subjects, 20–40% of the total platelet mass is pooled in the spleen, where they will typically spend up to one-third of their lifespan. The pooling and temporary sequestration that increase when the spleen is enlarged should be distinguished from the destruction of platelets in the spleen that occurs in many cases of thrombocytopenia.

Role of the spleen in ensuring quality control of red cells

The spleen is the body's largest filter of blood and a major function of the spleen is the quality control of red cells. Red cells are normally flexible and capable of transiting the splenic cords and entering the venous circulation, whereas cells with abnormal membranes, or those with inclusions that render them relatively inflexible, remain in the cords where they are repaired or destroyed. Sequestration is a reversible process whereby cells are temporarily trapped by adhesion to the reticular meshwork of the cords on their passage through the spleen. Phagocytosis is the irreversible uptake by macrophages of particulate matter, non-viable cells and viable cells that have been damaged by prolonged sequestration, antibody opsonisation or loss/alteration of surface CD47. In the presence of metabolically active macrophages, the densely packed red cells are deprived of oxygen and glucose. This stress increases membrane rigidity and reduces the natural deformability of the biconcave cell. This effect is particularly marked if there is an underlying abnormality of the red cell metabolic system, if cells are coated with antibodies or if they are fragmented or misshapen in other ways. In these situations, they remain trapped in the cord space and undergo phagocytosis. Siderotic granules, Howell–Jolly (DNA) bodies, nuclear remnants and Heinz bodies are removed by culling or pitting during temporary sequestration. After removal of the inclusions, the red cells return to the circulation. Reticulocytes may be retained in the splenic cords for a considerable proportion of the 2–3 days of their maturation period and during this time they lose their intracellular inclusions, alter the lipid composition of their surface and become smaller in size. It is not clear whether the spleen has any special role in the removal of normal aged red cells, and it seems more likely that such cells are removed by the general reticuloendothelial system, which includes both the spleen and the bone marrow.

Immunological function of the spleen

The spleen is the largest single accumulation of lymphoid tissue in the body and is estimated to contain 25% of the T-lymphocyte pool and 10–15% of the B-lymphocyte pool. T-cells, mainly of the $CD4^+$ subtype, are found predominantly in the periarteriolar lymphatic sheath, whereas B-cells are located in the follicles and marginal zones of the white pulp. There is also an abundance of macrophages and dendritic cells and the architecture is maintained through a complex anatomical organisation that includes a tubular conduit system that transports a range of chemokines and other molecules. The spleen has a unique role in acting as an immunological filter for the bloodstream. Lymphocytes and dendritic cells enter the splenic tissue by initial entry into the marginal zone and from there they can pass into the periarteriolar lymphatic sheath by crossing a lining of sinus cells. There is a constant flow of both T- and B-cells through the spleen, with T-cells typically staying in the spleen for a few hours, whereas B-cells stay in the follicle and marginal zone for prolonged periods. It remains unclear how lymphocytes exit the white pulp.

Of interest is the ability of the spleen to engage in both innate and adaptive immune responses. The marginal zone acts as a site for both of these processes, whereas the WP and RP are

largely restricted to adaptive and innate immune responses, respectively. Blood is filtered directly into the perifollicular zone, where the predominant cell populations are macrophages and marginal-zone B-cells. The macrophages express a range of pattern-recognition receptors that recognise bacterial molecules such as lipopolysaccharide. These cells thus act as an important filter for the clearance of blood-borne pathogens. The marginal-zone B-cells are a unique B-cell subset that bridges the innate and adaptive immune responses. They are able to recognise bacterial pathogens without the help of T-cells. This T-cell-independent antibody response consists largely of low-affinity IgM and can play an important role in limiting bacterial replication. In addition, these B-cells can act as antigen-presenting cells capable of migrating to the periarteriolar lymphatic sheath, where they present antigen to T-cells and facilitate the production of high-affinity IgG antibodies. B-cell maturation and clonal expansion occur in the follicles, with plasmablasts maturing into plasma cells that subsequently migrate to the RP, where they are retained through CXCR4/CXCL12 interactions.

The T-cell-independent antibody response outlined above produces a spectrum of low-affinity IgM antibody clones that provide a first line of immune defence against bacterial sepsis, especially from *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*. The spleen also appears to act as a defence against viral infections and intraerythrocytic parasitic infections such as *Plasmodium* and *Babesia*. The important role of the spleen in this regard is revealed by immunisation of splenectomised individuals with T-independent antigens, where antibody titres are typically only 10% of those seen in control subjects. Given these unique features, it is no surprise that splenectomy is associated with a degree of relative immunosuppression to encapsulated bacterial species.

Splenomegaly and hypersplenism

While splenomegaly refers to an anatomical increase in spleen size, hypersplenism refers to a functional syndrome characterised by an enlarged spleen; reduction of one or more cell lines in the peripheral blood; and hyperplastic cellularity of the bone marrow. Unsurprisingly, therefore, many of the underlying pathologies are common to both splenomegaly and hypersplenism.

Spleen size

The spleen is palpated in the supine or right lateral decubitus position. Light fingertip pressure is applied below the left costal margin during deep inspiration. A palpable spleen usually implies splenomegaly, although a normal-sized spleen may sometimes be palpable in children, adolescents and occasionally thin adults. In most adults, the spleen becomes palpable when its size is increased by around 40%. While it is an important clinical sign, identification of splenomegaly by means of a physical examination of the abdomen may be unreliable for a number of reasons. A mildly enlarged spleen may not extend far beyond the rib cage. Conversely, a massively enlarged spleen may extend so far into the pelvis that it is difficult to palpate its lower edge. Abdominal obesity or tense musculature may also make the spleen more difficult to palpate.

More reliable assessment for the extent of splenomegaly, and assessment for focal lesions is obtained by radiology through ultrasound, magnetic resonance imaging (MRI) and computed tomography (CT). The choice of imaging modality will depend on the reason for assessment and patient characteristics, with the calculated spleen size varying somewhat between methods. A deviation of 10–20% from the normal range is not considered significant unless there is a trend over time.

Causes of splenomegaly

Pathological enlargement of the spleen can occur in a wide range of conditions. The mechanism of enlargement varies according to the underlying aetiology, for example; reactive increase in WP in inflammation and infection; congestive expansion of RP with increased blood pool, cellular infiltration or extramedullary haemopoiesis. The relative incidence of each cause of splenomegaly is subject to geographical variation. In Western countries, leukaemia and lymphomas, myeloproliferative disorders, haemolytic anaemias, infectious mononucleosis and portal hypertension account for most cases. In tropical countries, the major causes are parasitic infections such as malaria, leishmaniasis and schistosomiasis. The more important haematological and non-haematological (systemic) causes of splenomegaly are provided in Table 5.1, some of which are discussed in more detail below.

Extramedullary haemopoiesis

Together with the liver, the spleen has a transient role in haemopoiesis *in utero* from the third month, continuing until birth (Chapter 31). From about 20 weeks gestation, the bone marrow becomes a site of haemopoiesis and this increases rapidly during the last trimester of pregnancy, whereas haemopoietic activity in the spleen disappears. There is no evidence for a specific inhibitor of splenic haemopoiesis and the spleen remains a potential site for blood production in the adult, particularly for the maintenance of erythropoiesis. This occurs during pathological states and the development of such extramedullary haemopoiesis after birth can occur as a compensatory erythroblastic hyperplasia in disorders of severe anaemia such as chronic haemolysis, megaloblastic anaemia, transfusion-dependent thalassaemias and some haemoglobinopathies e.g. HbC, HbE, HbSC and HbS/beta thalassaemia (HbSS is usually associated with splenic atrophy in adults although the spleen may be enlarged in children).

Table 5.1 Causes of splenomegaly.

Haematological
Acute leukaemia
Chronic myeloid leukaemia*
Chronic lymphocytic leukaemia*
Malignant lymphomas*
Myelofibrosis*
Polycythaemia vera*
Essential thrombocythaemia (some cases)
Hairy cell leukaemia*
Gaucher disease*, Niemann–Pick disease, Langerhans cell histiocytosis X
Primary splenic hyperplasia (i.e. unknown cause)
Thalassaemia
Sickle-cell disease, HbSC disease and other haemoglobinopathies**
Haemolytic anaemias*
Megaloblastic anaemia (rare)
Systemic
Acute infections: septicaemia, typhoid, infectious mononucleosis, cytomegalovirus
Subacute and chronic infections: tuberculosis, syphilis, brucellosis, subacute bacterial endocarditis, HIV
Tropical parasitic infections (tropical splenomegaly*): malaria*, leishmaniasis*, schistosomiasis*, trypanosomiasis
Collagen diseases: systemic lupus erythematosus, rheumatoid arthritis (Felty)
Sarcoidosis
Amyloidosis
Cysts
Haemangiomas
Carcinoma (rare)
Congestive splenomegaly
Portal hypertension*
Splenic/portal/hepatic vein obstruction
Congestive cardiac failure

* Splenomegaly is a frequent finding with these conditions.
** Sickle cell disease often causes an atrophic spleen later in life due to infarction.

Alternatively, extramedullary haemopoiesis may arise as a more generalised haemopoiesis often seen in primary myelofibrosis or other malignant disorders in the bone marrow, particularly those inducing significant marrow fibrosis. The mechanisms involved in this myelofibrosis-related extramedullary haemopoiesis are poorly understood; it is unclear if pluripotent stem cells are present in the spleen or migrate from the bone marrow. It is even conceivable that changes in the stroma or clonal haemopoietic stem cells driving the underlying pathological process are involved in favouring initiation of the extramedullary haemopoietic process.

Haemolytic anaemia and immune cytopenias

Haemolytic anaemias can be either predominantly intravascular or extravascular. In extravascular haemolytic anaemias such as warm autoimmune haemolytic anaemias, haemolytic transfusion reactions and red cell membrane defects such as hereditary spherocytosis and hereditary elliptocytosis, there is excess removal of red cells by the spleen and rest of the reticularendothelial system. This drives splenic enlargement through increased macrophage and cytotoxic T-cell proliferation and trapping of spherocytes within splenic sinusoids. Enhanced opsonisation of neutrophils or platelets, due to autoantibody production in immune thrombocytopenia purpura (ITP) and autoimmune neutropenia, also drives immune cell expansion of the red pulp.

Infections

Splenomegaly driven by infectious aetiologies (see Table 5.1) occurs due to a variety of factors including direct infiltration by the infective organism, reactive lymphoid change, and expanded phagocytic activity.

Infiltration by haematological malignancies

A comprehensive description of the various haematological malignancies that can infiltrate the spleen is beyond the scope of this chapter. However, some of the more commonly encountered and clinically important entities will be briefly considered.

Non-Hodgkin lymphoma

Non-Hodgkin lymphoma (NHL) infiltration of the spleen most commonly occurs in the setting of systemic lymphomatous spread. While NHL of various types can involve the spleen, it is particularly frequent in low-grade B-cell lymphomas. True primary splenic lymphoma in which disease is restricted to the spleen or splenic hilar lymph nodes is rare (accounting for less than 1% of all lymphomas). Dominant, although usually not isolated, splenic involvement is most frequently seen with splenic marginal zone lymphoma (SMZL). Small B-cell lymphomas including CLL/SLL, PLL, SMZL, LPL, HCL, FL and MCL typically cause fairly uniform nodular expansion of the WP with variable RP infiltration, while the formation of single or multiple discrete masses is more typical of DLBCL. The specific features of splenic involvement by T-cell and NK-cell malignancies are not well described, partly reflecting their rarity. However splenic involvement is characteristic of certain T-cell lymphomas with distinct clinicopathological characteristics. Hepatosplenic T-cell lymphoma and T-PLL both result in significant splenomegaly with diffuse infiltration of the RP but relative preservation of the cord and sinus architecture. In hepatosplenic T cell lymphoma (HSTL), there is marked reduction or near complete loss of the WP with variable WP involvement in T-PLL. T-LGL is also characterised by splenic involvement with

RP infiltration, but the WP is typically spared and patients often have less significant splenomegaly.

Hodgkin lymphoma

Although splenic involvement by classical Hodgkin lymphoma (cHL) is fairly common, primary disease is extremely rare. Splenic infiltrates are typically nodular and frequently present as distinct masses that may be multiple or solitary. As at other nodal sites, cHL in the spleen may be associated with prominent sarcoid-like granulomas and careful histological and immunophenotypic investigations is essential when cHL is suspected. Of note, granulomas in the absence of detectable cHL may be found in patients with an established cHL diagnosis.

Myeloid stem cell disorders

Although leukaemic disorders invariably involve the spleen, with neoplastic cells typically confined to the RP cords with secondary involvement of the sinuses, the extent of splenic involvement depends upon the type and duration of disease. Some chronic stem cell disorders frequently result in hypersplenism secondary to significant splenomegaly, but this is less commonly seen in de novo or rapidly emerging acute leukaemia. Splenomegaly in myeloproliferative neoplasm (MPN) is particularly well recognised with sudden changes in splenic size often a sign of acute transformation, classically blast crisis in chronic myeloid leukaemia (CML). Splenomegaly in PV reflects the stage and severity of disease, with intense RP congestion and cordal macrophage proliferation in the erythrocytotic phase of disease, followed by variable amounts of extramedullary haemopoiesis in the spent phase which is characterised by significant bone marrow fibrosis. Splenomegaly is most striking in primary myelofibrosis (PMF), driven by extramedullary haematopoiesis and may be a dominant presenting clinical feature at diagnosis. The splenic changes in ET are less well described and typically less pronounced than PV or PMF, with sudden or progressive enlargement suggestive of fibrotic progression or acute/blast transformation.

Infiltration by non-haematological malignancies and tumour-like lesions

The most common neoplasms of the spleen are vascular tumours. Splenic haemangiomas are relatively common benign vascular proliferation forming single or multiple tumour nodules; they are typically asymptomatic and discovered as incidental findings with only a minority resulting in splenomegaly or pain. Littoral cell angiomas are unique to the spleen and result in mild to moderate splenomegaly; they are regarded as benign with most cases successfully treated by splenectomy. Splenic angiosarcomas are aggressive, high-grade malignant neoplasms that typically present with splenomegaly, abdominal pain or splenic rupture. The prognosis is generally very poor, particularly when widely disseminated, with only rare cases of cure reported following splenectomy. Other splenic vascular proliferations or lesions with a prominent vascular component include lymphangioma, sclerosing angiomatoid nodular transformation of the spleen (SANT), peliosis and splenic hamartoma. Perhaps surprisingly, carcinoma of the spleen from metastasis is relatively rare although reports of microscopic tumour cell metastasis suggest their frequency may be underestimated by imaging studies. Carcinomas of the breast, stomach, lung and ovary are amongst the most common primary tumours to involve the spleen. Primary sarcomas of the spleen, with the exception of angiosarcoma, are extremely uncommon. Inflammatory pseudotumour-like dendritic cell sarcoma is peculiar to the spleen and liver, presenting with fever and abdominal pain and sometimes associated with other malignancies. This spindle cell tumour is positive for Epstein-Barr Virus (EBV) and expresses follicular dendritic cell markers; splenectomy is typically curative.

Though rare, patients with lysosomal storage diseases often demonstrate evidence of splenic involvement. Gaucher's and Niemann-Pick diseases are the most common storage diseases encountered in the spleen with hypersplenism sometimes resulting from significant splenomegaly. The pattern of RP expansion by numerous histiocytes is not specific for any lysosomal storage disorder. Other non-malignant causes of focal splenic lesions include abscesses, typically resulting from infective endocarditis or haematogenous seeding from other sites such as the respiratory or gastrointestinal tract or splenic infarcts. Splenic thromboemboli may be associated with atrial fibrillation; valvular heart disease or atrial septal defect; microvascular thrombi in sickle cell disease; or prothrombotic states such as antiphospholipid syndrome.

Vascular congestion

The spleen can become engorged with blood when there is increased vascular pressure secondary to parenchymal liver disease or congestive cardiac failure. The pathological mechanisms driving vascular congestion in parenchymal liver disease are multi-factorial and include portal congestion, altered haemodynamics and inflammatory mediators. Vascular obstruction due to portal, hepatic or splanchnic vein thrombosis can also cause splenic enlargement due to increased pressure. Vaso-occlusion in children with sickle cell disease can cause such rapid and profound engorgement of the spleen as to induce haemodynamic compromise. This is typically not seen in adults with sickle cell disease as the spleen is more frequently atrophic.

Diagnostic evaluation of splenomegaly and splenic lesions

Radiological or clinical evidence of splenomegaly should prompt careful evaluation of patients and consideration of the pathological processes/disorders summarised above. Clinical assessment for symptoms and risk factors along with appropriate laboratory

investigations (peripheral blood smear, viral serology, autoimmune serology, enzyme analysis, genetic studies, etc.) should be determined and prioritised based upon the initial differential diagnosis. Further imaging may be helpful, particularly for focal lesions. Abscesses, infarcts and cysts, can often be accurately characterised on CT and/or MRI. Contrast-enhanced CT/MRI is useful for suspected vascular lesions. PET-CT may be used to predict the likelihood of malignancy for solid lesions; PET avid lesions are more likely malignant or contain granulomas.

Biopsy of the spleen is now rarely performed due to the organ's extensive vascularity and the consequent high risk of bleeding. Even for those patients with suspected malignant infiltration of the spleen, alternative sites for tissue biopsy are preferable. In cases of suspected haematological malignancy, bone marrow aspirate and trephine, lymph node biopsy or flow cytometry of peripheral blood are often sufficient for diagnosis. Where other alternative modalities have failed or there is no other potential site for tissue diagnosis, CT-guided biopsy of focal lesions may be indicated. Diagnostic splenectomy is usually reserved as a last resort for patients in whom a diagnosis cannot be reached in any other way, malignancy is suspected, and tissue diagnosis is essential to guide management. In some cases, where there is diagnostic uncertainty and the patient does not need urgent treatment, such as for suspected asymptomatic low-grade lymphoproliferative disorder, expectant management potentially with serial imaging may be appropriate.

Management of splenomegaly

Appropriate management of splenic enlargement depends on the underlying cause, with treatment potentially reducing spleen size and leading to symptomatic improvement.

Splenectomy

Splenectomy is rarely performed for symptomatic splenomegaly as other measures are often effective, and it is associated with an increased risk of infection and thrombosis, as well as entailing perioperative risk. However, it may be appropriate in some specific cases where other interventions have been unsuccessful or are inappropriate, such as hereditary spherocytosis or immune thrombocytopenia or even occasionally in malignant conditions such as SMZL where the bulk of the tumour population is located in the spleen. It may also sometimes be used for diagnosis of unexplained splenomegaly, as described above. The role of splenectomy in the individual diseases described above is discussed in the relevant chapters. For elective splenectomy, a laparoscopic approach is preferred where possible because of reduced morbidity and mortality.

Splenectomy is now most commonly performed in the emergency setting following splenic rupture. Splenic injury is common after blunt abdominal trauma and must be assessed in order to consider the need for emergency splenectomy. Focused abdominal sonography for trauma (FAST) is a form of ultrasound that is rapid and particularly useful in patients who are haemodynamically unstable. Abdominal CT remains the investigation of choice when the pulse and blood pressure remain stable. Low-grade injury may be managed conservatively, and angioembolisation can be very useful for controlling haemorrhage while preserving splenic function (Figure 5.2). Atraumatic splenic rupture may also occur but is rare, most commonly resulting from neoplastic haematological infiltration, infection (especially EBV) or drug/treatment-related (e.g. granulocyte colony-stimulating factor).

Where splenectomy is contemplated in a planned capacity, the preoperative evaluation of the patient requires close co-operation between surgeon and haematologist. Vaccinations should be performed and it is valuable to check liver function, obtain appropriate imaging and potentially evaluate the hepatic and portal blood flow by Doppler ultrasound examination. Haemoglobin and platelet counts should be optimised which may be particularly challenging in cases of autoimmune cytopenias and may require pre-operative interventions such as intravenous immunoglobulin.

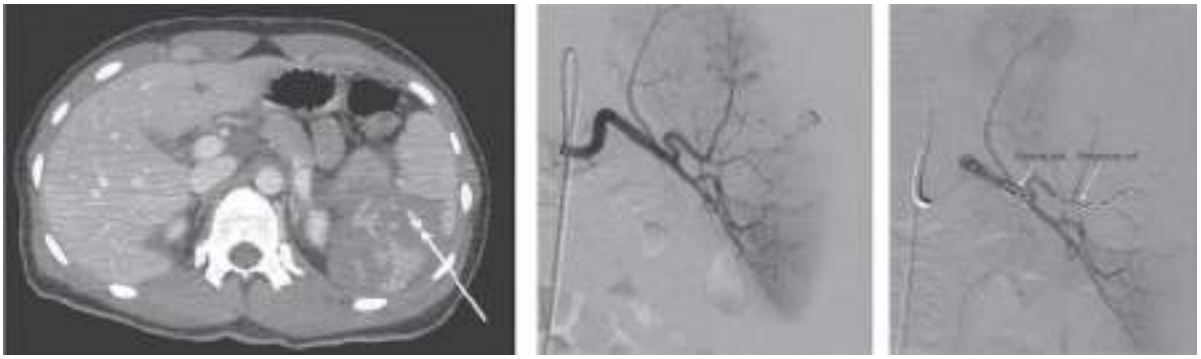

Figure 5.2 (a) Computed tomogram showing splenic injury (Organ Injury Scale grade 4) with contrast extravasation (white arrow). (b) Angiography (i) with signs of ongoing bleeding before embolisation and (ii) after distal and proximal embolisation (Source: Skattum *et al.* (2012)/Reproduced with permission of John Wiley & Sons.)

In some cases, simultaneous cholecystectomy may be performed if there is a history of gallstones which can develop as a complication of chronic haemolysis.

Complications of splenectomy

Bleeding can be a complication after surgery and usually comes from the peritoneal and diaphragmatic surfaces rather than from identifiable blood vessels. Frequently, no specific bleeding source is found at re-operation. Venous thromboembolism (VTE) risk is increased post splenectomy and is likely to be multifactorial, including thrombocytosis, which peaks at round 7–12 days post splenectomy and persists indefinitely due to alterations in the vascular anatomy and changes in coagulation parameters. The risk of VTE is particularly high in patients with hereditary stomatocytosis and splenectomy is avoided for this reason. Close attention should be paid to VTE prophylaxis, evaluation of signs and symptoms suggestive of VTE, and patient education on discharge.

There is some evidence of an association between cardiovascular events and pulmonary hypertension and splenectomy. However, convincing evidence for a causal relationship is lacking. Indeed, pulmonary hypertension may in fact be secondary to chronic haemolysis for which the splenectomy was originally performed. Some case series have reported an increased risk of cancer following splenectomy, although such an association has not been consistently reproduced. Accessory splenic tissue, an anatomically separate collection of splenic tissues thought to arise following failed tissue fusion during embryogenesis, may be overlooked at operation; after splenectomy, it may enlarge and cause a recurrence of the symptoms for which the original operation was performed.

Post splenectomy sepsis

Splenectomy increases the risk of developing an overwhelming bacterial infection, (sometimes termed *overwhelming post splenectomy sepsis*; OPSS), which in historical cohorts can be fatal in up to 50% of patients. The onset is often acute and fulminant with a non-specific presentation that may include fever, muscle ache, chills, sore throat, diarrhoea or vomiting. Septicaemia or meningitis can be seen, and features such as disseminated intravascular coagulation and hypoglycaemia may develop rapidly. *S. pneumoniae* is the most common cause and bacteraemia can be so severe that cocci are seen directly in stained blood films. *H. influenzae*, *N. meningitidis* and *Escherichia coli* may also be implicated. The underlying cause is a defective reticuloendothelial clearance of encapsulated organisms, combined with delayed and impaired development of the IgM antibody response. Infection by encapsulated bacteria is therefore the greatest concern, although infections are not limited to encapsulated organisms. The risk of infection is partially correlated with the underlying disease for which splenectomy was performed (see Figure 5.3).

Infection may occur at any age, but children are at increased risk, particularly within the first few years of life. Splenectomy should therefore be postponed until after the age of 5 years of age wherever possible. The prevention of fatal post splenectomy sepsis has four components:

- Patient education
- Vaccination
- Antibiotic prophylaxis
- Early empirical use of antibiotics for febrile episodes.

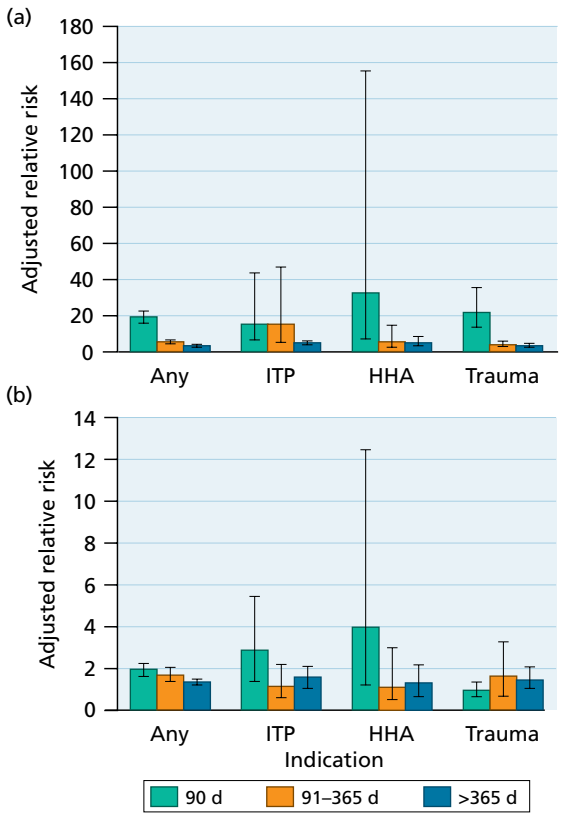

Figure 5.3 Relative risk of hospital contact for any infection in splenectomised patients according to the time since splenectomy. Data is based on a Danish population-based study (Thomsen *et al.* 2009). Results are shown for: any indication of splenectomy, immune thrombocytopenia, hereditary haemolytic anaemias and trauma, in comparison with age- and sex-matched subjects from the general population (a) and up to five matched-indication non-splenectomised patients (b) (Source: Rodeghiero and Ruggeri (2012). Reproduced with permission of John Wiley & Sons.)

Patient education

Education of patients or parents is perhaps the most important aspect of management, to ensure that they are aware of the possibility of infection and know how to react appropriately. Patients should be advised to carry a card or bracelet to alert health professionals to the potential risk of overwhelming infection. When travelling to tropical areas, asplenic patients are at

increased risk of severe *Plasmodium* infection and must adhere scrupulously to antimalarial prophylaxis. Patients should also be advised that severe infection can occur after animal bites, typically due to *Capnocytophaga canimorsus* which is a commensal bacterium of cats and dogs.

Vaccination

When splenectomy is being planned the patient should be considered for immunisation against pneumococcus, *H. influenzae* type B (Hib) and meningococcal infection. To obtain the maximum immune response, patients should be immunised at least 2 weeks and ideally 10–12 weeks before splenectomy. Vaccines, including live inoculations, can be given safely after splenectomy and appropriate vaccines that were not administered before splenectomy should be given 14 days post splenectomy. One approach to vaccine policy for hyposplenic patients is shown in Table 5.2.

S. pneumoniae is the major concern and there are two major forms of vaccine available. Polyvalent polysaccharide pneumococcal vaccines (PPV) include a wide range of different serotypes, typically at least 23, but their efficacy in hyposplenic individuals is poor. Conjugated vaccines consist of a bacterial polysaccharide linked (conjugated) to a carrier protein and thereby enlist a T-cell immune response. The 13-valent pneumococcal conjugate vaccine (PCV13) is more effective than PPV in hyposplenic and asplenic patients, although the breadth of serotype coverage is not as great. The optimal schedule of vaccination with both subtypes is not yet known and, in some countries, PCV vaccines are not recommended, whereas others suggest a schedule such as PCV13 followed 2 months later by PPV23. The recommendations for vaccination in children under 5 years will depend on the prior vaccination history (Table 5.2). Annual influenza vaccine is also recommended for asplenic or hyposplenic patients, primarily as influenza may predispose patients to secondary bacterial infection.

Antibiotics

Long-term antibiotic prophylaxis should also be taken into consideration but there are some concerns about antibiotic resistance especially if there is poor compliance. There is a

Table 5.2 Suggested schedule for vaccine immunisation in individuals with asplenia or splenic hypofunction.

Age at which asplenia or splenic dysfunction acquired	Vaccination schedule*		
	Month 0	**Month 1**	**Later**
Under 2 years	Complete according to national routine childhood schedule, including booster doses of Hib/MenC and PCV13	A dose of MenACWY conjugate vaccine should be given at least 1 month after the Hib/MenC and PCV13 booster doses	After the second birthday, one additional dose of Hib/MenC and a dose of PPV should be given
Over 2 years and under 5 years (previously completed routine childhood vaccinations with PCV7)	HibMenC booster PCV13	MenACWY conjugate vaccine	PPV (at least 2 months after PCV13)
Over 2 years and under 5 years (previously completed routine childhood vaccinations with PCV13)	HibMenC booster PPV	MenACWY conjugate vaccine	
Over 2 years and under 5 years (unvaccinated or previously partially vaccinated with PCV7)	HibMenC vaccine First dose of PCV13	MenACWY conjugate vaccine	Second dose of PCV13 and then PPV (at least 2 months after PCV13)
Over 5 years (regardless of vaccination history)	HibMenC vaccine PVV	MenACWY conjugate vaccine (boosters every 5 years)	

* Where possible, vaccination course should ideally be started at least 2 weeks before surgery or commencement of immunosuppressive treatment PCV, pneumococcal conjugate vaccine; PPV, pneumococcal polysaccharide vaccine.
(*Source:* Davies *et al.* (2011) *Br. J. Haem.* **155**: 308–17. Reproduced with permission of John Wiley & Sons.)

relatively poor evidence base for this approach in the era of pneumococcal vaccination and different regimens are sometimes advised. The British Society for Haematology advises prophylaxis for patients at high risk of infection. These are defined as:

- Age less than 16 years *or* greater than 50 years
- Inadequate serological response to pneumococcal vaccination
- History of previous invasive pneumococcal disease
- Splenectomy for underlying haematological malignancy, particularly in the context of ongoing immunosuppression.
- The immediate postoperative period is when risks are highest.

Those at lower risk should be advised about the risks and benefits of lifelong antibiotics and may wish to discontinue them. Oral penicillin 250 mg b.d. is usually recommended, and patients who are allergic to penicillin should be offered erythromycin 250 mg b.d. Pre-emptive antibiotics should be given to patients so that they may start them if they develop a fever or infective symptoms such as pyrexia, malaise or shivering and cannot get to hospital within 2 hours. In all such cases, the patient should also seek immediate medical help. Signs of systemic infection in an asplenic individual should be considered a medical emergency and most patients should be admitted to hospital and started on broad-spectrum antibiotics after cultures have been taken, pending specific antibiotic sensitivity results.

Changes in peripheral blood following splenectomy

Red cell changes

The changes in red cell morphology include the presence of Howell–Jolly bodies, siderotic granules and target cells. In a proportion of subjects, irregularly contracted or crenated acanthocytic forms are also a feature (Figure 5.4). There is usually an increase in the number of reticulocytes in the circulation and occasionally isolated erythroblasts are seen. However, there is no alteration in red cell survival.

Leucocyte changes

After splenectomy, there is a rise in the total leucocyte count. A neutrophil leucocytosis in the immediate postoperative period is later replaced by a significant and permanent increase in both lymphocytes and monocytes. Usually, the total white cell count stabilises at between 10 and 15×10^9/L, but occasionally it may rise to twice this level. Minor increases in blood eosinophils and basophils have been noted after splenectomy, but this is not a regular feature. In response to infection, splenectomised subjects produce a much greater leucocytosis than people with intact spleens. Often, there is a marked left shift in the differential leucocyte count, with myelocytes and occasionally more primitive cells.

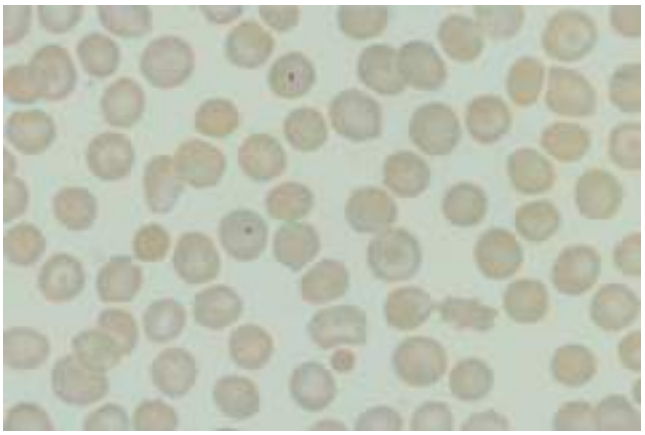

Figure 5.4 Blood film showing features of hyposplenism: Howell–Jolly bodies, target cells and contracted cells.

Platelet changes

As indicated above, the thrombocytosis after splenectomy is usually transitory and falls to normal or near-normal values over the following 1–2 months. However, even if the platelet count has returned to normal values, occasional large and bizarre platelets can be seen in the blood films of many splenectomised subjects. Their presence suggests that the spleen normally removes these particular platelets.

Hyposplenism

Absent or reduced splenic function occurs in a range of medical conditions, as well as following surgical splenectomy or therapeutic splenic embolisation. In some disorders such as sickle cell disease, severe gluten-induced enteropathy and untreated human immunodeficiency virus (HIV) infection it is a fairly frequent complication. It may also be seen less frequently in a variety of other conditions (see Table 5.3). In sickle cell anaemia, there is functional asplenia by 1 year of age, and autoinfarction leads to a state of anatomical asplenia after 6–8 years of age. Congenital absence of the spleen is rare and may be associated with organ transposition and severe malformations of the heart and lungs. At the other extreme of life, atrophy of the spleen is seen after around 65 years of age, with a rapid decrease in weight in older age. Patients with functional hyposplenism have impaired immunity to blood-borne bacterial and protozoal infections and persistent thrombocytosis. Management is similar to that required after splenectomy, including prophylactic antibiotics and vaccines (see above) and advice to seek medical attention immediately in the event of illness or fever.

Table 5.3 Causes of hyposplenism.

Congenital aplasia syndrome
Ageing
Haematological disorders
Sickle cell disease
Thrombocythaemia
Myelofibrosis
Malaria
Lymphomas
Circulatory
Splenic arterial/venous thrombosis
Autoimmune disease
Systemic lupus erythematosus
Rheumatoid arthritis
Hyperthyroidism
Sarcoidosis
Chronic graft-versus-host disease
Combined immunodeficiency
Gastrointestinal (? immune basis)
Gluten-induced enteropathy
Dermatitis herpetiformis
Crohn's disease
Ulcerative colitis
Tropical sprue
Infiltrations
Lymphomas
Sézary syndrome
Myeloma
Amyloidosis
Secondary carcinomas, especially breast
Cysts, e.g. hydatid
Nephrotic syndrome
Drugs
Methyldopa
Intravenous immunoglobulin
Corticosteroids
Irradiation
Splenectomy and splenic embolisation

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CHAPTER 6

Cancer immunotherapy

6

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Introduction

A relationship between the immune system and cancer was first hypothesised in the late nineteenth century. Anton Chekhov, a famous playwright, reported on the association between infection and cancer remission, writing 'It has long been noted that the growth of malignant tumours halts for a time when this disease [(erysipelas)] is present'. William Coley, a surgeon in New York, re-enforced this concept by demonstrating that an immune response induced through injection of *Streptococcus pyogenes* bacilli could improve cancer patient outcomes, cementing a relationship between the immune system and cancer.

At the beginning of the 20th century, the concept of 'cancer immunosurveillance' was formed. This hypothesis, mainly curated by Lewis Thomas and Sir Frank Macfarlane Burnet, stated that the immune system can detect and target tumour-associated neoantigens (Oiseth and Aziz 2017). Extensive data supported their hypothesis, with immune-deficient knockout murine models demonstrating the relationship between an immune deficiency and unfavourable cancer outcomes (Waldman *et al.* 2020). In 2011, Hanahan and Weinberg revised their hallmarks of cancer to include loss of immune surveillance.

Our knowledge of the interactions between the immune system and cancer has since grown exponentially. This has culminated in the development of the field of immunotherapy, where the immune system is utilised to survey, target and fight cancer, being recognised as the fifth pillar of cancer treatment.

Cancer immunotherapy is a broad term encompassing a wide range of various technologies from antibody and cytokine-based drugs, through to cellular therapies (see Figure 6.1). One of the key paradigm-changing developments has been in the field of checkpoint inhibitors (CPI); monoclonal antibodies (mAbs) that disrupt inhibitory signals allowing restoration of robust immune surveillance in the presence of established cancer (Robert 2020). The discovery of cluster of differentiation antigen-152 (CD152)/cytotoxic T-lymphocyte associated protein-4 (CTLA4) and the CD279/programmed cell death protein-1 (PD-1), CD274/programmed death ligand-1 (PD-L1) co-inhibitory biology earned James P. Allison and Tasuku Honjo the Nobel Prize in Physiology or Medicine in 2018. Blockade of these receptors for clinical development has led to remarkable responses with durable survival for a subset of haematological and non-haematological cancer patients.

Immunotherapy is key to the treatment of an ever-increasing list of malignancies. However, it does not come without its disadvantages. Loss of peripheral tolerance and triggering of auto-inflammation complicates the use of CPIs and potentially fatal complications of cellular therapies remain relatively common (Waldman *et al.* 2020). In addition, they are not always effective, and the results of treatment with CPIs in myeloid malignancies, for instance, were not encouraging. Therefore, selecting the right type of therapy and optimum timing for each individual cancer type/patient is crucial.

This chapter will describe the immune evasion and subversion in cancer, immunotherapeutic strategies to counteract this

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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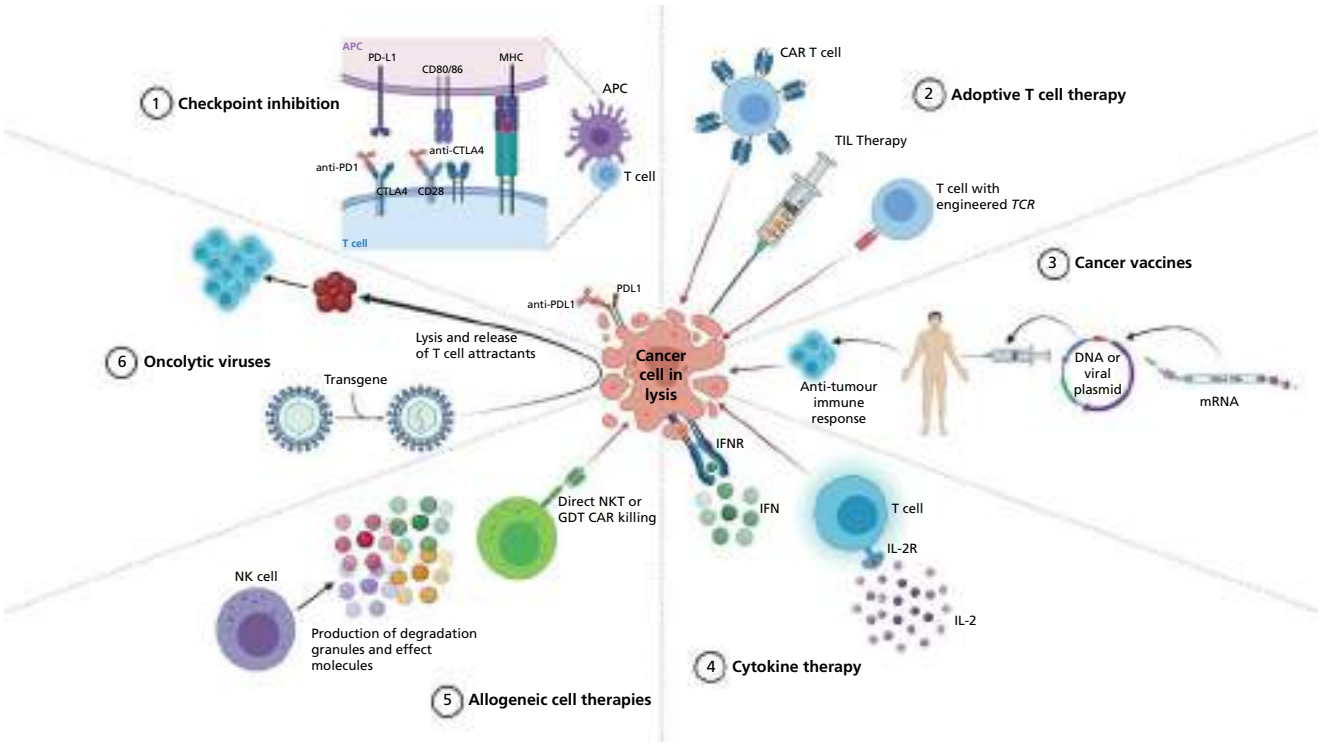

Figure 6.1 Immunotherapeutic approaches to the treatment of cancer. 1. Checkpoint inhibition therapy: where monoclonal antibodies target and neutralise receptors such as CTLA4 on T cells and ligands such as PDL1 on cancer cells. 2. Adoptive T cell therapy: includes CAR T cell therapy, TIL therapy and *TCR* gene transfer therapy. 3. Cancer vaccines, mRNA is loaded into plasmids and injected into the patient to raise an anti-tumour response. 4. Cytokine therapy: includes the use of IL-2 which stimulates T cell proliferation, as well as IFN which has direct anti-cancer activity. 5. Allogeneic cell therapies: NK cells can be used to produce degradation molecules or attract T cells, NKT and GDT CARs can be used for direct killing of cancer cells. 6. Oncolytic viruses: virus is engineered to lyse tumour cells as well as incite an immune response. APC, Antigen Presenting Cell; CAR, Chimeric Antigen Receptor; CTLA4, Cytotoxic T-Lymphocyte Associated Protein 4; GDT, Gamma Delta T Cell; IFN, Interferon; IFNR, Interferon Receptor; IL-2: Interleukin-2; MHC, Major Histocompatibility Complex; NK, Natural Killer Cell; NKT, Natural Killer T cell; PD1, Programme-Death Receptor 1; PDL1, Programme-Death Ligand 1; TIL, Tumour Infiltrating Lymphocyte.

subversion and immunotherapy limitations. We will finish with an outlook on the future of immunotherapy with its progression towards centre stage in cancer treatment. Immunotherapy strategies in specific haematological malignancies are discussed separately in relevant chapters.

Immune evasion and subversion in cancer

The immune system and tumour microenvironment

The human immune system is comprised of two systems, the innate and the adaptive. The innate system encompasses physical barriers and cells that are the first responders to invading antigen, including dendritic cells, macrophages, eosinophils, basophils, neutrophils, mast cells and natural killer cells. The adaptive immune system responds more specifically and forms immunological memory. It comprises B cells, $CD4^+$ and $CD8^+$ T cells, unconventional T cells (γδ T cells, NKT cells and MAIT cells) and innate lymphoid cells.

The environment in which a tumour resides is known as the tumour microenvironment (TME) (Anderson and Simon 2020) (see Figure 6.2). It is where most of the cancer cell and immune system interactions happen and is composed of a complex collection of blood vessels, immune cells (innate and adaptive), stromal cells and extracellular matrix. The composition of the TME depends in part on the type of tissue the tumour grows in (Anderson and Simon 2020).

The adaptive immune system utilises different cell types to continuously balance its fight against threat whilst sustaining self-tolerance and suppressing excessive inflammation. This is exemplified by the juxtaposition of cytotoxic $CD8^+$ T cells that detect and destroy transformed cells and T regulatory cells (Tregs) which are the immune system's most suppressive cells.

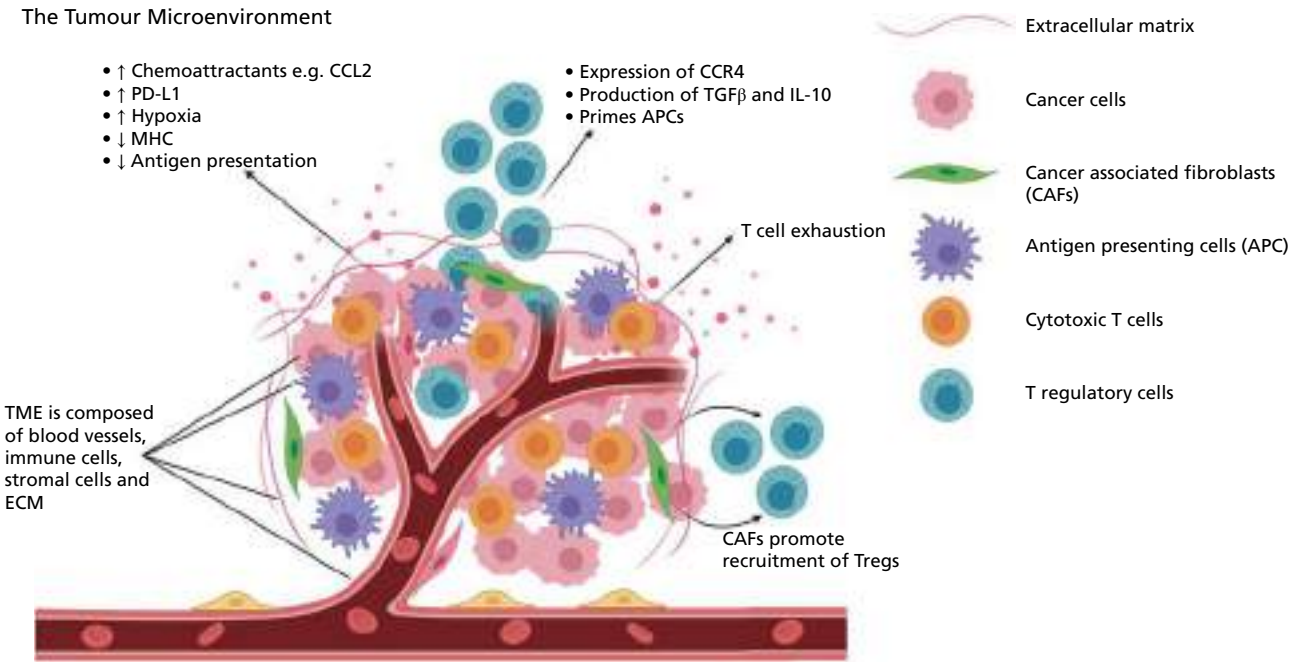

Figure 6.2 The Solid Tumour Microenvironment (TME) is a highly immunosuppressive environment composed of a large array of immune cells, stromal cells, cancer cells, extracellular matrix and blood vessels. Arrow pointing up, Upregulation; Arrow pointing down, Downregulation; APC, Antigen Presenting Cell; CCL2, Chemokine Ligand 2; CCR4, Chemokine Receptor 4; IL-10, Interleukin-10; MHC, Major Histocompatibility Complex; PDL1, Programme Death Receptor 1; TGFb, Transforming Growth Factor beta.

Within the TME, Tregs are unfavourable as they can promote tumour growth and reduce anti-tumour response. Indeed, the TMEs can demonstrate multiple mechanisms used to escape $CD8^+$ T cell cytotoxicity and enhance the suppressive activity of Tregs (Vinay *et al.* 2015). One primary mechanism is the secretion of Treg-attractant chemokines such as C-C Motif Chemokine Ligand 22 (CCL22) by cancer cells, such as ovarian cancer cells. CCL22 is recognised by C-C Chemokine Receptor Type 4 (CCR4) which is expressed on Tregs. When CCL22 is secreted by the cancer cells, it induces the migration of Tregs to the TME, resulting in the suppression of immune response against the cancer. Cancer cells can additionally secrete factors that induce the proliferation of Tregs, such as transforming growth factor β (TGFβ) and interleukin 10 (IL-10). Once attracted to the TME, Tregs can prime antigen-presenting cells to capture costimulatory molecules CD80/86 in a CTLA4-dependant manner in a process known as trans-endocytosis. After the removal of CD80/86, co-stimulation is inhibited, thus rendering T cells anergic and dysfunctional. Additionally, specific Treg subsets are shown to be able to provide a 'niche' for stem cells in animal model. Whether these Tregs could support a malignant clone in the microenvironment is yet to be determined.

In addition to enhancing the suppressive capabilities of Tregs, cancer cells have been shown to down-regulate the expression of their major histocompatibility complex (MHC); the antigen-displaying molecules. Cancer cells are derived from normal self-cells and therefore most of the peptides that they express are minimally recognised by T cells. However, mutations within the cancer cells lead to the formation of neoantigens. These are highly immunogenic antigens and are not expressed in healthy tissue, making them highly attractive targets for immunotherapy (Zhang *et al.* 2021). However, this can be inhibited by the cancer cell's ability to downregulate their MHC, meaning that the neoantigens are not displayed, thus evading recognition and cytolysis by T cells.

Another mechanism by which cancer cells supress the immune response is by inducing unresponsiveness and dysfunction in T cells, a state known as T cell exhaustion (Wherry and Kurachi 2015). Tumour cells can upregulate ligands that bind to T cells and induce exhaustion, thereby rendering the T cells unable to fight the cancer (Wherry and Kurachi 2015). One such ligand that cancer cells can express is PD-L1. The binding of PD-L1 to PD1 inhibits intracellular T cell signalling which restrains the immune response. The docking of PD-L1 induces the phosphorylation of PD1's two intracellular tyrosines and recruitment of SH2-domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2. These become dephosphorylated thereby preventing the phosphorylation and activation of downstream effectors CD3ζ and Zeta Chain of TCR-Associated Protein 70

(ZAP70) which are important in early T cell activation. PD-L1 is naturally expressed by immune cells including macrophages and dendritic cells and it binds the PD1 receptor on T cells. The binding of PD-L1 to PD1 inhibits intracellular T cell signalling which restrains immune response. This interaction occurs naturally to prevent T cell hyperactivation and to keep the immune response in check. However, when cancerous cells overexpress PD-L1, it prevents T cells from being able to mount a response against the cancer.

There are also physical barriers to the infiltration of T cells into the tumour. As previously mentioned, the TME is a complex interplay of cells and extracellular matrix. It is a highly hypoxic environment that not only leads to increased malignancy, but also inhibits the activity of T cells. The TME is also a 'stiff' environment, which promotes tumour formation and inhibits the migration of T cells to the cancer site. For instance, cancer-associated fibroblasts (CAFs) suppress the immune response by promoting the recruitment of Tregs.

Reviving an effective immune response against malignant cells is the main focus of immunotherapy. This can be achieved through several approaches which are discussed in the next sections.

Types of immunotherapy

Antibody therapy

Immunotherapy aims to reintroduce malignant cell recognition and destruction. Efforts have been made to develop therapies that can home to the cancer and produce a localised response rather than a systemic one. Such therapies include the use of mAbs. These are proteins produced by B cells that are used to neutralise foreign antigens such as an invading virus or bacteria. They can be engineered to selectively target tumour-associated antigens (TAAs) and elicit an immune response (Reichert and Valge-Archer 2007).

For instance, Rituximab, an anti-CD20 antibody, that induces antibody-dependent cellular cytotoxicity (ADCC) towards B cells, is now routinely used in the treatment of lymphomas. They can also be engineered to target factors such as PD1 without engaging cellular cytotoxicity to overcome T cell exhaustion, allowing T cell activation and reintroduction of an immune response against the cancer (Reichert and Valge-Archer 2007).

Three signals are required to activate T cells antigen recognition, co-stimulation and cytokine signals (see Figures 6.3 and 6.4).

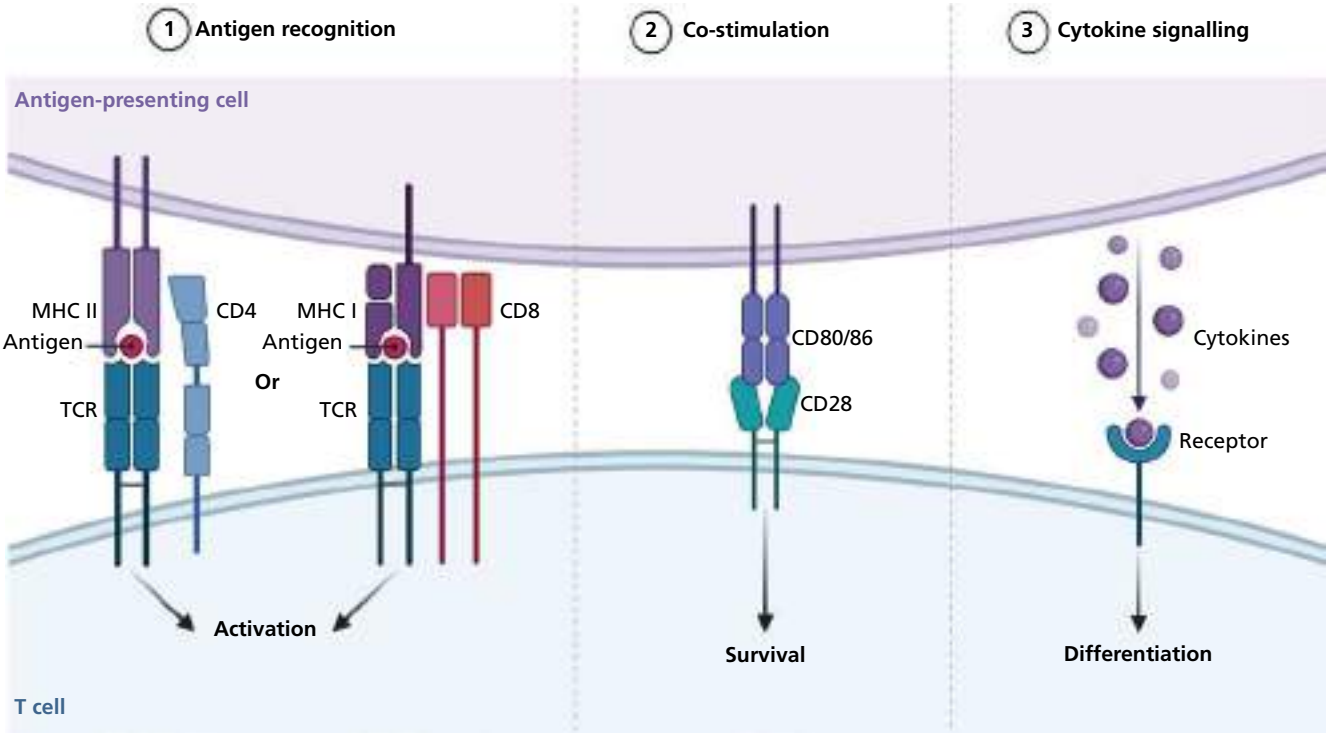

Figure 6.3 Three Signals of T cells activation. 1. Antigen recognition occurs when the APC presents an antigen via either MHC I or II to the TCR of the T cell, this is known as signal one. 2. Co-stimulation is known as signal two and involves the binding of molecules on the APC such as CD80/86 to the CD28 receptor on the T cell, this activity aids T cell activation. 3. Cytokine signalling induces the differentiation of the T cell into either effector or regulatory phenotypes and is known as signal three. APC, Antigen Presenting Cell; MHC, Major Histocompatibility Complex; TCR, T Cell Receptor.

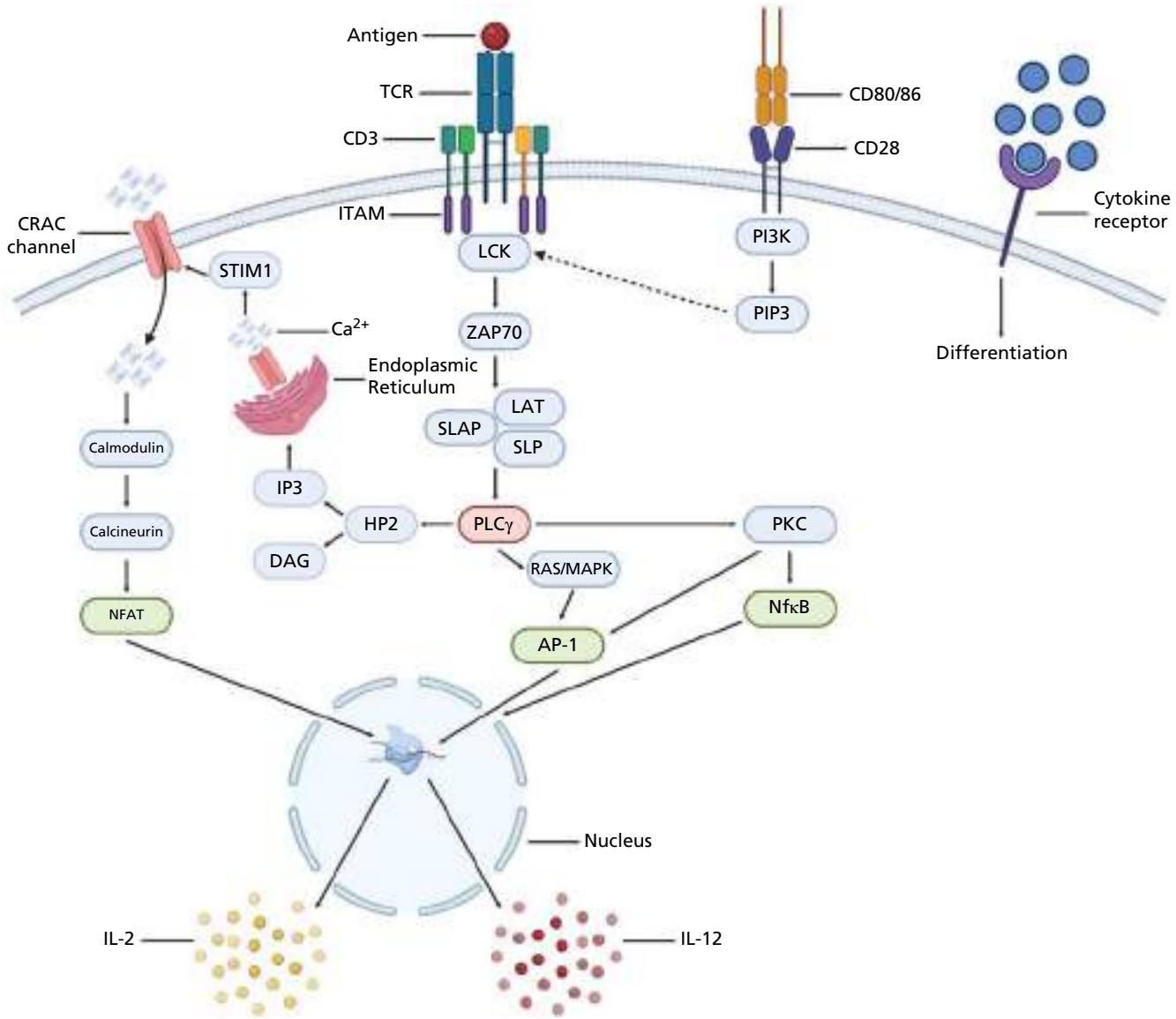

Figure 6.4 T cell activation: The first signal of T cell activation is TCR engagement with antigen. This initiated a signal which is transmitted by co-receptor CD3 via ITAMS which are phosphorylated by LCK. This phosphorylation provides a binding site for ZAP70 which phosphorylates adaptor proteins SLAP, SLP and LAT, resulting in the recruitment and activation of PLC-g. This has three different effects; the release of calcium, the activation of the RAS/MAPK pathway and the activation of PKC, all of which lead to the activation of different transcription factors involved in the activation of the T cell. The release of calcium begins with PLC-g cleaving PIP2 into IP3 and DAG. IP3 binds to calcium receptors in the ER membrane which releases calcium this stimulating STIM1 to open the CRAC channels allowing calcium flow into the rest of the cell. The increased intracellular calcium causes a conformational change in calmodulin which activates calcineurin. Calcineurin dephosphorylates NFAT which moves to the nucleus and acts as a transcription factor, inducing the production of IL-2. PLC- g also activates the RAS/MAPK pathway which activates AP-1 and subsequence genes similar to NFAT, inducing IL-12. Finally, PLC- g activates PKC which activated transcription factors NfkB and AP-1. The second signal of T cell activation, co-stimulation, occurs when CD28 comes in to contact with one of two molecules on the APC, CD80 or CD86. Engagement of CD28 induces tyrosine phosphorylation which activated PI3K with subsequent production of PIP3. This results in the recruitment of enzymes such as LCK which are needed for the activation of PLC-g. Stimulation from cytokines such as IL-12 act as the third signal and instruct how the T cell differentiates. AP-1, Activator Protein 1; CRAC, Ca^{2+}release-activated Ca^{2+}; DAG, diacylglycerol; IP3, Inositol triphosphate; ITAMS, Immunoreceptor Tyrosine-Based Activation Motifs; LAT, Linker for Activation of T cells; LCK, Lymphocyte-Specific Protein Tyrosine Kinase; NFAT, nuclear factor of activated T cells; NFkB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; PIP2, Phosphatidylinositol 4,5-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; PI3K, PI-3 kinase; PKC, Protein Kinase C; PLC-g, phospholipase C gamma; RAS, a small GTPase; MAPK, Mitogen-activated protein kinase; TCR, T Cell Receptor; ZAP70, Zeta Chain of TCR-Associated Protein 70; SLAP, Src-like adaptor protein; SLP, Src homology 2 (SH2) domain-containing leukocyte protein.

The immune system has several mechanisms in place to prevent T cell hyperactivation and autoimmunity. After T cell activation, a protein receptor called CTLA4 is expressed on the surface of the T cell. It competes with CD28 for CD80 and CD86, thereby preventing co-stimulation and inhibiting further T cell activation. Similarly, when an APC is presenting an antigen to a T cell, it simultaneously expresses PD-L1/2, which binds to PD1/2 receptor on the T cell. PD1 was identified as a mediator of apoptosis in 1992 by Honjo and colleagues. The docking of PD-L1 or PD-L2 inhibits intracellular cytokine signalling, which in turn restrains T cell activity.

The discovery that CTLA4 and PD1 negatively regulate T cell activation led to the idea that blocking these receptors could be used as a therapeutic against cancer. This idea was first tested by James Allison and colleagues in 1996 and since, mAbs that target immune checkpoints, termed checkpoint inhibitors, have been widely developed and have revolutionised cancer therapy. These act by directly binding to and neutralising the checkpoint molecules, preventing them from inducing T cell exhaustion.

Anti-CTLA4

The CTLA4-based therapy ipilimumab was the first checkpoint immunotherapy (CPI) to receive FDA approval for unresectable stage III/IV melanoma in 2011. This came after a trial demonstrated ipilimumab treatment to confer a 3.6-month short-term survival benefit in metastatic melanoma patients when combined with glycoprotein 100 (gp100) peptide vaccine, compared to patients receiving gp100 alone (hazard ratio for death, 0.68; $P < 0.001$). More impressive still, the long-term survival meta-analysis data showed that 22% of melanoma patients treated with ipilimumab gained an additional 3 years of life compared to treatment-naïve patients.

It is thought that anti-CTLA4 works not only by preventing the inactivation of cytotoxic T cells, but also by inducing the death of immunosuppressive Treg cells. CTLA4 is highly expressed in Treg cells. When bound to the Fcγ receptor on APCs, anti-CTLA4 can induce ADCC against the Tregs that it binds. This would be disadvantageous against cytotoxic T cells, however, it is useful when directed at Treg cells.

Anti-PD1

As previously discussed, the PD-L1-PD1 interaction is a key mechanism used by the immune system to prevent T cell hyperactivation, keeping the immune response in check. Preclinical studies showed that blockade of PD-L1 or PD1 not only enhances anti-tumour response but limits metastasis in mouse models. The first anti-PD1 mAbs, pembrolizumab and nivolumab, were FDA-approved for the treatment of refractory and unresectable melanomas in 2014. Nivolumab showed in one phase III-controlled study to yield an overall rate of survival of 72.9% in the nivolumab-treated group compared to 42.1% in patients treated with chemotherapeutic dacarbazine (hazard ratio for death, 0.42; 99.79% CI, 0.25–0.73; $P < 0.001$).

Pembrolizumab also demonstrated significant results. One trial investigating anti-PD1 treatment in non-small cell lung cancer, showed Pembrolizumab treatment to provide 10.3 months of cancer-progression-free survival compared to 6 months in the chemotherapy-treated patient group (hazard ratio for disease progression or death, 0.50; 95% CI, 0.37–0.68; $P < 0.001$).

Since 2014, Nivolumab and Pembrolizumab have been FDA-approved for the treatment of multiple forms of cancer.

Recently, other checkpoint inhibitors/negative regulators of T cell activation have been investigated as new targets for checkpoint blockade, including Lymphocyte-Activating Protein 3 (LAG3), T cell Immunoglobulin (TIM3), V-Domain Ig Suppressor of T cell Activation (VISTA), B7 Homolog three protein (B7-H3/CD276) and T cell Immunoreceptor with Ig and ITIM Domains (TIGIT). In particular, Relatlimab, an anti-LAG3 therapy, in combination with anti-PD1 mAb Nivolumab, has recently shown to confer a significant progression-free survival benefit compared to Nivolumab monotherapy in patients with advanced melanoma.

Combination therapies

Due to the success of CPI therapy, investigations have taken place in combining them to yield even more favourable responses. In 2017, data from a phase III trial showed metastatic melanoma and renal cell carcinoma patients to have increased survival when administered with ipilimumab in combination with nivolumab compared with ipilimumab alone (hazard ratio for death with nivolumab plus ipilimumab vs. ipilimumab, 0.55 [$P < 0.001$]; hazard ratio for death with nivolumab vs. ipilimumab, 0.65 [$P < 0.001$]) (Wolchok *et al.* 2017). However, adverse events were experienced in 96% of patients treated with the combination therapy, compared to 86% in the monotherapy treatment group. In addition, these adverse events were reported to be severe or life-threatening (grade 3 or 4) in 59% of combination therapy-treated patients compared to 21% in the nivolumab-only group and 28% in the ipilimumab-only group. Despite these toxicities, stable to complete response was reported in 70% of combination-treated patients, compared to 54% in nivolumab and 41% in ipilimumab-treated patients. These results have delivered practice-changing progress in survival for melanoma patients over the past decade. Further work to develop novel combinations and to 'de-risk' toxicity is ongoing.

Similarly, a combination of radiotherapy and both anti-PD1 and anti-CTLA4 have shown promising preclinical results and await further investigation in clinical trials. In addition, radiotherapy in

combination with checkpoint blockade has been shown to enhance the abscopal effect when treatment at one site leads to metastatic regression at another site.

Adverse effects of checkpoint inhibition

Despite the successes of Ipilimumab, Nivolumab and Pembrolizumab, these therapies do not come without adverse effects. Blockade of inhibitory checkpoints can lead to auto-inflammation and auto-immune reactions. This professes itself through the accumulation of hyperactive memory T cells which travel to peripheral organs and cause inflammation. The most common immune-related adverse events (irAEs) include fatigue, skin manifestations such as pruritis, gastrointestinal inflammation, hepatic toxicity and endocrine dysregulation. Targeting CTLA4 is associated with higher rates of irAEs and combination therapy with both anti-CLTA4 and anti-PD1 therapy further increasing the risk of toxicity. Less commonly arthritis, nephritis, uveitis and pneumonitis can occur. In fact, any organ can be affected, and occasionally, toxicity can be life-threatening. The management of irAEs requires immune suppression to dampen down an out-of-control auto-reactive immune state.

In addition to the adverse effects of checkpoint inhibition, one-quarter to one-third of metastatic melanoma patients become resistant to therapy and relapse over time, despite initially having objective responses and receiving continuous therapy. This is due to a combination of intrinsic and extrinsic factors including T cell exhaustion and the downregulation of tumour antigen presentation. Studies are currently investigating whether combinatorial therapies can overcome these resistance mechanisms.

Whilst checkpoint inhibition does not guarantee complete remission and can yield adverse effects, it has proven revolutionary in the treatment of cancer, with those treated gaining significant extensions of life. Future work is needed to reduce associated toxicities and elucidate the mechanisms of tumour resistance to CPI.

Antibody-drug conjugates

While chemotherapy is still the main approach in the majority of malignancies, the cytotoxic effect is the limiting factor and causes various side effects. Thus, more targeted chemotherapy approaches have long been desired. Antibody-Drug Conjugate (ADC) is one of the approaches, which has been employed to achieve a more targeted drug delivery and less systemic toxicity. ADCs are developed by combining a tumour antigen-specific antibody and cytotoxic agent. Gemtuzumab ozogamicin (Mylotarg) consists of a cytotoxic antibiotic (calicheamicin) and a recombinant monoclonal antibody against the CD33, is an example of ADCs, which is used for the treatment of acute myeloid leukaemia (AML).

Bispecific antibodies

mAbs have shown great success in the clinic but are restricted by their monospecific targeting. One type of therapy that has shown promise to circumvent such specificity is the use of small bivalent antibody fragments known as diabodies or bispecific antibodies (bsAbs) (Ahamadi-Fesharaki *et al*. 2019). These antibodies have two antigen binding Fc domains which can simultaneously target two antigens involved in the same cancer or infection. Since 2009, two bsAbs have been approved for clinical use against EpCAM-positive carcinoma and relapsed or refractory acute lymphoblastic leukaemia, and a further 60 diabodies are under investigation in clinical trials (Ahamadi-Fesharaki *et al*. 2019). For instance, Mosunetuzumab is an anti-CD20 -CD3 bispecific antibody (Ab) that redirects T cells to eliminate malignant B cells. This novel treatment has shown in clinical trial to be effective in patients with Relapsed/Refractory Follicular Lymphoma who have received $\geq$2 prior lines of therapy.

Adoptive T cell therapy

In recent years, adoptive T cell (ATC) therapy has shown promising results in cancer patients. This form of immunotherapy involves modifying autologous or allogenic T cells and infusing them into cancer patients. A study demonstrating tumour regression after leukocyte transfer first appeared in 1966 by Southam and colleagues. Later, and initially unintentional, donor lymphocyte infusions appeared as one of the first forms of ATC. Kolb and Colleagues demonstrated that lymphocytes administered to patients with acute or chronic leukaemia in relapse after bone marrow transplantation mediated a graft-versus-tumour effect against leukaemic cells, reducing tumour burden. Since these early studies, three main types of ATC therapies have emerged including *T cell receptor* (*TCR*) gene therapy, ATC therapy with tumour-infiltrating lymphocytes (TILs) and ATC with synthetic Chimeric Antigen Receptors (CARs).

Tumour-infiltrating lymphocyte therapy

ATC therapy using TILs first appeared in the late 1980s (Rosenberg *et al*. 1979) and involves isolating TILs from a patient tumour biopsy, expanding the cells with IL-2 and reinfusing the cells into the patient where they can produce a potent immunological response against the tumour. Studies have shown this therapy to work more efficiently when the patient is depleted of lymphocytes (lymphodepletion) prior to

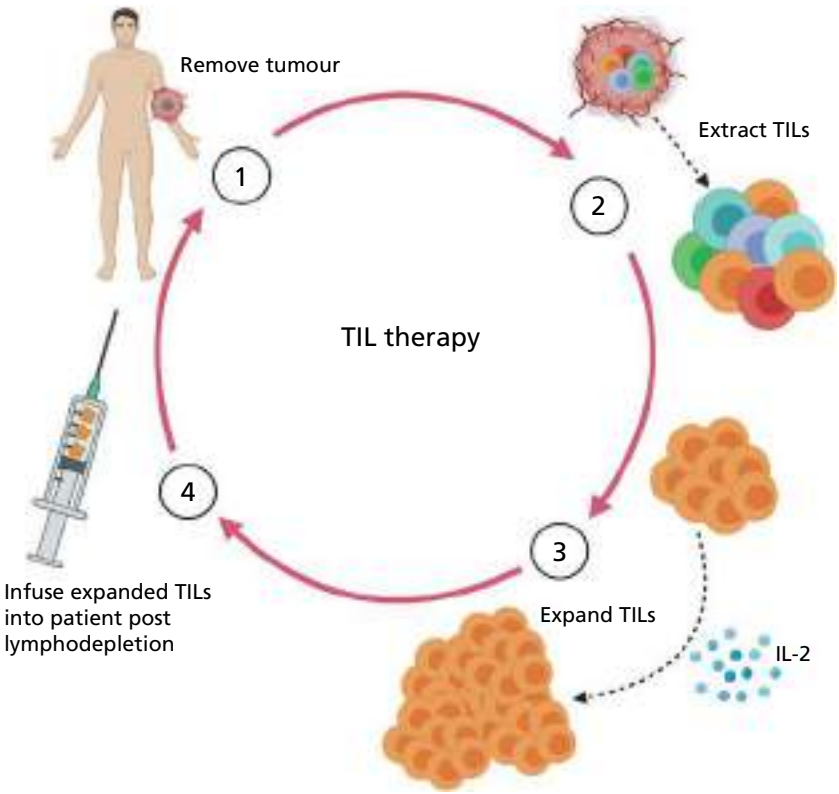

Figure 6.5 TIL therapy: 1. Tumour is removed from the patient. 2. TILs are extracted from the tumour. 3. TILs are expanded with IL-2. 4. Expanded TILs are re-infused into patients post lymphodepletion conditioning. TIL, Tumour Infiltrating Lymphocyte; IL-2, Interleukin-2.

the infusion, with studies showing complete objective response rates of up to 72% and tumour regression in up to 24% of treated metastatic melanoma patients. Recently, TIL therapy in combination with up to six doses of IL-2 has shown promise in patients with advanced melanoma who have progressed after checkpoint inhibition and targeted therapies. A phase II trial involving lymphodepleted patients who had received a mean of 3.3 prior treatments demonstrated objective response rates of up to 81% after receiving TIL infusions. However, melanomas are one of the only cancer types to reliably give rise to TILs with anti-tumour activity. Furthermore, TILs must be grown *in vivo* for this therapy to work and produce a long-lasting response (see Figure 6.5), ensuing a long production process when time is not always available.

T cell receptor gene therapy

Studies using TILs spawned the idea of modifying the T cell receptor (TCR) in order to increase the specificity of response against the tumour. This form of therapy is called *TCR* gene therapy and involves genetically modifying the *TCR* to recognise specific cancer antigens, eliminating the need to extract/produce and expand TILs. When the modified *TCR* binds the antigen plus MHC of interest, such as NY-ESO-1, it induces T cell activation and anti-tumour activity. Recently, the first CRISPR-edited *TCR* gene therapy showed safety in a phase I clinical trial, presenting *TCR* gene therapy as a promising technology (Stadtmauer *et al.* 1979). However, the downside to this type of therapy is that the T cells require antigen presentation from APCs in order to become activated. The reliance on other cell types has prompted research into bypassing MHC antigen presentation, leading to the creation of CAR T cells.

CAR T cell therapy

CARs are synthetic structures that consist of four main components, an antigen-binding domain, a hinge region, a

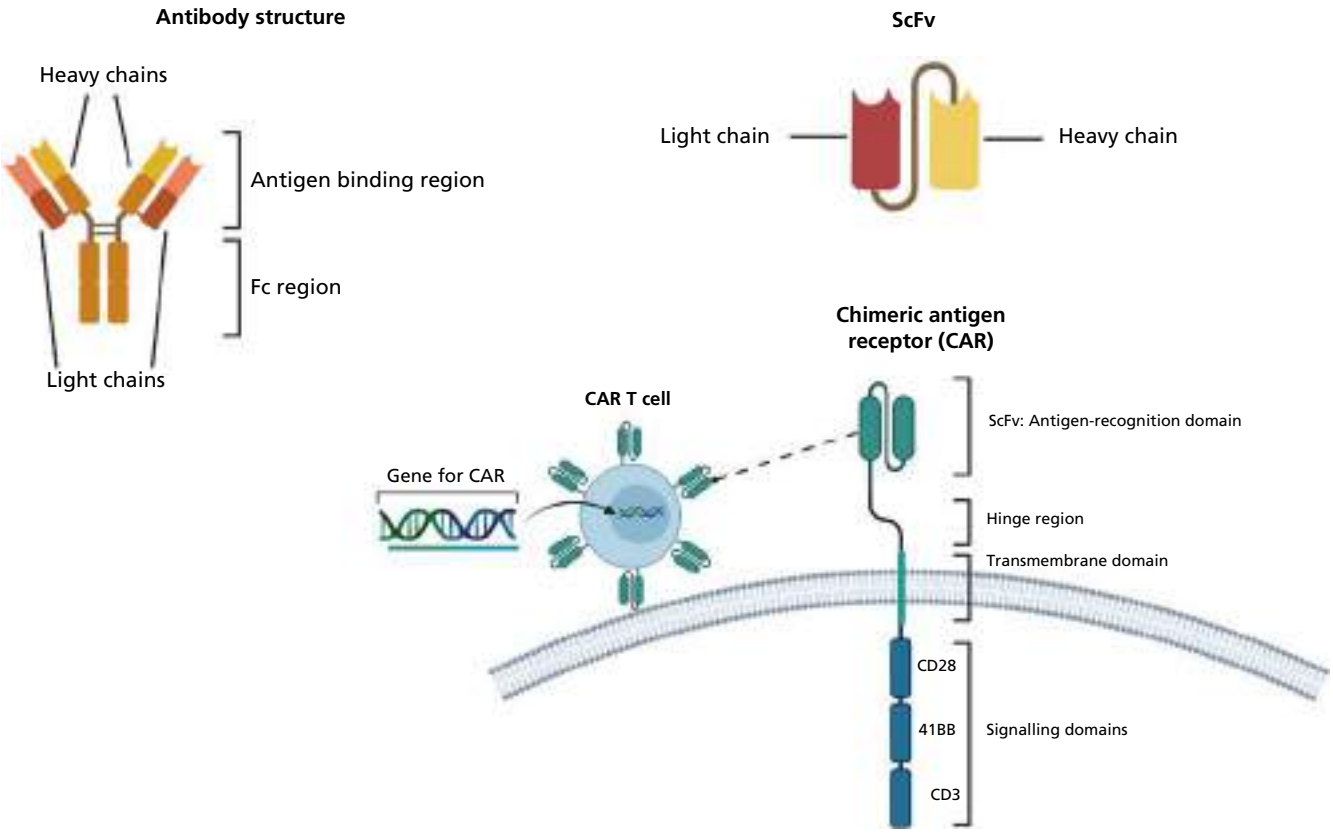

Figure 6.6 Antibody structure: Composed of a light and heavy chain forming the antigen binding region. The Fc region interacts with Fc receptors on the surface of immune cells and elicits signalling. Scfv are engineered structures originating from antibodies and used to generate CAR T cells. CARs consist of four main components, an antigen-binding domain, a hinge region, a transmembrane domain, and an intracellular signalling domain. CAR, chimeric antigen receptor; Fc, fragment crystallisation; ScFv, single chain variable fragment.

transmembrane domain and an intracellular signalling domain (see Figure 6.6).

The antigen binding domain is commonly composed of a variable heavy (Vh) and light (Vl) chain derived from a monoclonal antibody, connected via a linker, forming what is known as a single chain variable fragment (ScFv) (Sterner and Sterner 2021). It is the ScFv that is coupled to a CD3-singalling domain which targets extracellular cancer antigens and provides signal 1 of T cell activation. The hinge connects the ScFv to the transmembrane domain which anchors the CAR to the T cell membrane. Below this is the intracellular domain, where co-stimulatory molecules such as CD28 or 4-1BB are inserted and used to initiate intracellular signalling within the T cell, providing signal 2 of T cell activation. Fourth-generation CARs have additional domains including chimeric cytokine receptor 4αβ which when docked by IL-4, induces the production of IL-2 and further T cell proliferation, providing signal 3 of T cell activation.

The generation of CAR T cell therapies is an extremely precise science and is largely due to the combined efforts of immunologists, bioengineers and geneticists. It is thanks to this multidisciplinary research that five CAR T cell therapies have been brought to clinic since 2017. The first FDA-approved CAR was directed at CD19, an antigen expressed on B cells, for the treatment of relapsed/refractory acute lymphoblastic leukaemia (ALL). CD19 CARs have shown great success, with initial studies showing complete remission in relapsed B-acute lymphoblastic leukaemia patients and a 5-year follow up again showing complete remission in 83% of patients. The largest impact to date for CD19-directed CAR T cells however has been in relapsed/refractory diffuse large B-cell lymphoma where three products have now received an FDA license. The latest CAR, Idecabtagene vicleucel, was approved for use against relapsed or refractory multiple myeloma in March 2021. As CD123, an IL-3 receptor subunit, is expressed on CD34+CD38- AML blasts, it is considered a potential target for CAR therapy in AML as well as other surface proteins such as CD33.

Limitations and adverse effects of ATC therapy

A limitation of CAR T cell therapy is the reliance on finding TAAs for the CARs to target. The CD19 antigen, for instance, is not a TAA, it is an expendable antigen. Normal B cells expressing CD19 are also killed by the CAR T therapy, something that can be managed with supportive therapies.

Whilst CAR T cell therapy has proven effective in the treatment of haematological malignancies, its use against solid tumours has proven less successful to date. This is primarily due to the lack of cancer-specific antigens which allow for the migration of CAR T cells to the tumour site (Sterner and Sterner 2021). Current CAR T cell therapy research is therefore highly focused on the discovery of neo-antigen cancer targets. The most promising targets under investigation include Glutamate Carboxypeptidase 2, also known as Prostate-Specific Membrane Antigen (PSMA) and Fibroblast Activation Protein Alpha (FAP). Antigen heterogeneity in tumours is another barrier to effective CAR T cell therapy. Tumour cells display a large array of antigens, facilitating escape from the CAR T cell response. Efforts have been made to increase CAR specificity to multiple antigens, however, this can lead to an increase in off-target toxicity. As previously mentioned, the TME is a hypoxic and 'stiff' environment where collagen-rich stroma can prevent T cell infiltration. This is especially enhanced in solid tumours. In addition, the TME is highly immunosuppressive, partially due to the presence of Tregs and expression of antigens such as PD-L1, which can impair the function of T effector cells and enhance T cell exhaustion (Sterner and Sterner 2021). With more than 90% of cancer-related deaths in America being due to epithelial solid tumours, combating these barriers could prove revolutionary in the treatment of cancer.

It is important to consider that like checkpoint inhibition therapy, the use of TILs and CAR T cell therapy can yield severe adverse effects, the most common being cytokine release syndrome (CRS). CRS occurs when the T cell becomes activated and releases inflammatory cytokines. This is a natural reaction by the T cell but is amplified in ATC therapy due to the amount of T cells being activated. Clinical features of CRS include fever, myalgias, malaise, loss of appetite and muscle pain. Another adverse event of ATC therapy is neurotoxicity, including Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS) and CAR T cell-related encephalopathy, manifestations of which can include seizures, cerebral oedema, headaches, confusion, ataxia, delirium and aparaxia. In addition, lymphodepletion and the use of IL-2 in TIL therapy are associated with additional toxicities. IL-2 treatment can lead to capillary leak syndrome which can present itself as oedemas, hypotension reduced urination, myalgia, fever and nausea. Toxicities associated with lymphodepletion consist largely of pancytopenia and neutropenia, as well as thrombocytopenia and lymphopenia.

ATC therapy has opened many doors to treat a wide range of cancers. To date, the most successful CARs have targeted specific antigens. The future of CAR T cell therapy will likely involve focusing on solid tumour cancers and myeloid malignancies such as myelodysplastic syndrome (MDS) and AML, increasing the specificity to multiple tumour-specific antigens (TSAs) without increasing toxicities, as well as engineering the CARs to survive better within the immunosuppressive TME. Associated toxicities remain the biggest barrier to the widespread implementation of CAR T cell therapy.

Cancer vaccines

Cancer vaccines are employed to prompt the immune system to respond and fight the malignant cells. Two main types exist, prophylactic and therapeutic vaccines. These vaccines are largely in the form of synthetic peptides or messenger RNA (mRNA) that are loaded into a DNA or viral plasmids. These plasmids are injected into the patient to yield an immune response against the injected peptide or mRNA.

Prophylactic

Prophylactic vaccines are generally administered to healthy individuals to prevent cancer. They can be used, for instance, to prevent infection from oncogenic viruses. It is estimated that globally, 13% of cancers are due to chronic viral, bacterial or parasitic infections. Therefore, targeting these infections can lead to reduced incidence of cancer.

Hepatitis B (HBV) is the leading cause of global liver cancer incidence. Prophylactic HBV vaccines have shown great success in reducing the incidence of hepatocellular carcinoma (HCC), especially when administered early in childhood. One study in Taiwan, showed an 80% decline in HCC incidence in cohorts born after the country's HBV vaccination programme began.

Like HBV, human papillomavirus (HPV) is oncogenic. It is causatively associated with head and neck cancer and cervical cancer. A study in Sweden demonstrated that administration of HPV vaccines, especially before the age of 17, can substantially reduce the risk of cervical cancer (Lei *et al.* 2020). Of the over 1.5 million girls and women who took part in the study, only 19 women who had received a HPV vaccine were diagnosed with cervical cancer, as opposed to 538 women who had not received the vaccine (Lei *et al.* 2020).

Prophylactic vaccines against oncogenic viruses have, therefore, proven successful in the prevention of cancer. There is a great demand for worldwide vaccination programmes against HPV and HBV, with studies such as the one in Taiwan playing a vital role in this implementation. However, there remain other viruses that require attention, including Epstein-Barr Virus (EBV). This is another virus aetiologically linked with

the onset of various cancers. First discovered in cells cultured from Burkitt Lymphoma, EBV is widespread in all human populations, but infection is largely asymptomatic. EBV infection can, in rare cases, become activated in immunodeficient patients and transform B cells into fast-growing lymphoblastoid cell lines (LCLs). It is thought that EBV is associated with 200,000 cases of cancer annually, yet no vaccine is currently available against EBV. Few vaccines are in phase I clinical trials, including a multiplication-incompetent-attenuated pox viral vector of modified vaccinia virus Ankara (MVA) encoding EBV antigens EBNA1 and LMP2. This vaccine has been shown to be well tolerated, however, the efficacy is yet to be shown. Considering the strong association between EBV infections and cancer, the development of an EBV vaccine is a high priority.

Therapeutic

Cancer vaccines can also be therapeutic, meaning they are administered to directly trigger anti-tumour immunity. Historically, these types of vaccines involved administering patients with their own tumour cells together with a virus to activate the immune response against the TAAs (Waldman *et al*. 2020). This method has seen the rise of vaccines such as Provenge; a vaccine formulated to target prostate acid phosphatase (PAP) for the treatment of metastatic castration-resistant prostate cancer, FDA-approved for clinical use in 2010. However, targeting TAAs such as PAP can elicit auto-immune and off-target responses. There has therefore been a push to discover TSAs that can produce a more effective and localised response to the tumour. As described in Section 6.2, cancerous cells often undergo mutations that lead to the production of novel self-antigens called neoantigens. These are not expressed in healthy tissue and can be recognised by T cells. Thanks to the development of next-generation sequencing, these tumour-specific mutations can be identified. For instance, Sahin and colleagues found over 1680 mutations in murine colon carcinoma cells, 48 of which were taken forward for further investigation due to their specificity and immunogenicity. Of these 48, 16 were made into mRNA vaccines and were found to generate an immune response in the vaccinated individuals, producing a strong anti-tumour response. Therapeutic vaccines are now largely based on targeting these neoantigens forming what is now called personalised recombinant cancer vaccines. *Wilms tumor 1* (*WT1*) gene product peptide vaccination has also been tried in MDS and AML with some encouraging results and could elicit antigen-specific $CD8^+$ T cell response in these patients.

Rapid advances in genomics and data science are likely to push this novel form of immunotherapy forward. However, there are time delays and high costs associated with the manufacturing of personalised vaccines that target an individual's specific TSAs. Efforts to reduce these barriers have so far involved improving vaccine delivery platforms. There is also research taking place on when best to administer other therapies in conjunction with cancer vaccines to improve clinical outcomes.

Cytokine therapy

Cytokines are a group of peptides, proteins or glycoproteins that are produced by immune cells. They act as signalling molecules that modulate and regulate immune cells and the immune response. Due to their ability to potentiate immune responses, they have been investigated as a form of immunotherapy, where administration could induce immune responses against cancer. Indeed, pro-inflammatory cytokines were found to increase the number of effector T cells within the TME as well as enhance T cell cytolytic activity (Berraondo *et al*. 2019). Two cytokines, IL-2 and Interferon α (IFNα), have proven particularly successful.

IL-2 is a key cytokine in the proliferation and survival of T cells and is used to expand T cells for ATC therapy. The first metastatic melanoma patient to be administration IL-2 had a complete response and was disease-free for 29 years. Further trials showed IL-2 administration to yield objective response rates of up to 20% in patients with metastatic renal cell carcinomas. The other successful cytokines are interferons (IFN). These are some of the most potent initiators of signalling cascades within the immune system, utilisation of which has shown tumour regression in over a dozen types of cancer, including myeloma, Karposi's sarcoma, melanoma, lymphoma and renal cell and bladder carcinoma.

The efficacy of IL-2 and IFN treatment led to their approval by the FDA for clinical use in the late 1990's against a wide range of cancers such as advanced renal cell carcinoma, metastatic melanoma, hairy cell leukaemia, follicular non-Hodgkin lymphoma and AIDS-related Kaposi's sarcoma (Berraondo *et al*. 2019). Despite this success, cytokine therapy has several limitations. The first being the severe toxicities that they can cause. Considering the vast effects that cytokines have on the immune system, namely their ability to induce pro-inflammatory activity, administration of potent cytokines can cause a range of haematologic, pulmonary, endocrine, neurologic, dermatological and autoimmune adverse events. Another limitation is the short half-life of cytokines and their sub-optimal localisation to the tumour (Berraondo *et al*. 2019). However, alterations to the cytokine structure can be made to increase the half-life. For instance, IFNα can be pegylated to protect it from proteolytic breakdown, thereby increasing the cytokine's half-life. Additionally, IFNα can be fused to apolipoproteins to increase the cytokine's anti-tumour activity and pharmacokinetics.

Although recombinant cytokine therapy has largely been superseded by CPIs, there is potential for future development through further engineering. Approaches in development include immune-cytokine design where a cytokine is linked to an antibody to deliver tumour specificity and engineering approaches to extend cytokine half-life (Berraondo *et al*. 2019).

Emerging types of immunotherapy

Oncolytic viruses

When a myelogenous leukaemia patient experienced tumour remission after an influenza virus infection, it hatched the idea of using viruses as a therapy against cancer. This discovery, in the early 1900's, led to the concept of oncolytic viruses (OV). OVs are viruses engineered to either lyse tumour cells whilst sparing healthy cells or to incite the immune system to raise an anti-cancer immune response (Zheng *et al*. 2019). In October 2015, the first herpes-derived OV, talimogene laherparepvec (T-Vec), was approved by the FDA for the treatment of advanced melanoma.

Recently, a novel combination therapy has emerged where patients are treated with both OVs and checkpoint inhibition. Several trials are underway, namely involving the combination of OVs such as T-Vec, HF-10, ONCOS-102, Pexa-Vec and a form of checkpoint inhibition. Results have been promising, with one phase I study showing objective response rates of 50% in T-Vec + ipilimumab-treated melanoma patients, with limited toxicity. Another trial of T-Vec + pembrolizumab for the treatment of melanoma has shown similar results, with objective response rates of up to 62%, again maintaining tolerable and limited toxicities. OVs can also be combined with CAR T cell therapy and cancer vaccines.

One of the main barriers to this therapy is the effective delivery of the OVs to the tumour site, as systemic administration of the OVs can result in premature degradation of the viral construct by the immune system. Future work will focus on editing the capsid of the virus to make it 'unrecognisable' to the immune system, and to develop novel carriers, such as transformed cells and tumour cell-derived microparticles to deliver the OV to the tumour.

Allogeneic cell therapies

NK cells

Natural Killer (NK) cells are immuno-surveillant, cytolytic cells belonging to the innate lymphoid cell family. They are activated through a set of stimulatory and inhibitory receptors; therefore, their activation depends on the balance of activating and inhibitory signals present. Once activated, they detect whether the adjacent cells are targeted for killing and induce cytolysis through the release of degradation granules (Shimasaki *et al*. 2020). As well as degrading target cells, NK cells can release multiple effector molecules including IFNγ, Tumour Necrosis Factor (TNF) and CC-chemokine ligand 4 (CCL4) which can modulate and magnify the immune response from other cells (Shimasaki *et al*. 2020). Their ability to modulate the immune response has been investigated as a form of therapy. Studies have shown NK cells to attract effector T cells to the tumour site and enhance the inflammatory response against the tumour. They have also been shown to eliminate circulating tumour cells and prevent the formation of metastasis.

NK CAR therapy

The development of CAR T cell therapy and its clinical success has paved the way for the development of other forms of CAR therapy. In particular, NK cells have been investigated as potential cells with which to express CARs. Studies utilising NK CARs have presented encouraging results, with one, in particular, showing that 64% of treated patients with either non-Hodgkin lymphoma or chronic lymphocytic leukaemia, had a complete remission, without the development of toxicities. There are multiple advantages for using NK cells over traditional T cells for CAR therapy. As hinted in the above study, the primary advantage is the reduced toxicities experienced with NK CARS, as NK cells release cytokines that are less likely to cause CRS and neurotoxicity compared to those released by T cells. By inhibiting the cytokine-inducible Src homology 2–containing (CIS) protein, it is also possible to further enhance the fitness of CAR-NK cells and their tumour cell-killing function. In addition to the engineered CAR-directed tumour killing, NK cells are naturally primed to direct cytolysis against tumour cells through the process of immunosurveillance (Xie *et al*. 2020). Finally, NK CARs have reduced risk of causing graft versus host disease. Therefore, they could represent an 'off the shelf' allogeneic cell therapy, rather than the personalised and costly traditional autologous CAR T cell therapy.

Other cells under current investigation for use as CAR therapies include NKT cells, macrophages and gamma delta T cells.

Future of immunotherapy

Now a standard pillar of cancer treatment, immunotherapy has proven to be specific, durable and effective. Checkpoint inhibition, adoptive T cell therapy, cancer vaccines, cytokine therapy and emerging forms of immunotherapy have all shown encouraging preclinical results with most having been FDA-approved for clinical use.

Despite the high enthusiasm for these forms of cancer treatment in some haematological malignancies, there remain barriers in the treatment of solid tumours, owing to the complexities of the solid TME. Another issue requiring attention is the varying patient response to immunotherapy and the development of resistance to treatment (Sharma *et al*. 2017). This can be explained by the continuously changing TME and interaction between the immune system and cancer cells. Tumour cells are genomically unstable and can produce tumour-cell intrinsic factors that prevent immune-mediated eradicaton (Sharma *et al*. 2017). Due to their instability, cancer cells may not always express the same antigens, thereby facilitating the escape from immune recognition and response (Sharma *et al*. 2017). In addition, extrinsic factors are also at play such as the presence of immunosuppressive cells (Sharma *et al*. 2017).

Advances are currently underway in improving patient selection for treatment (Iivanainen and Koivunen 2020) and in determining the intrinsic and extrinsic factors involved in resistance. Unveiling these mechanisms will not only be vital in overcoming resistance, but also in improving the prediction of treatment efficacy. Biomarkers may also provide a useful tool in predicting how resistant a patient is prior to treatment. Currently, the best markers in solid tumours include total tumour mutational load and density and distribution of $CD8^+$ T lymphocytes (Sharma *et al*. 2017), which may not be directly applicable to many haematological malignancies. Therefore, there is an unmet need to identify predictive biomarkers for response to immunotherapy in the majority of haematological malignancies (Sharma *et al*. 2017). Further to better understanding the TME, it is also necessary to research the molecular and cellular interactions that regulate the immune response in order to optimise efficacy and reduce toxicity.

The future of immunotherapy will also involve reevaluating the costs of these treatments with an aim to decrease the per-patient expense. This is necessary as CAR T cell therapies such as tisagenlecleucel can cost up to US$475,000 per patient, not including the adverse effects of treatment costs. Checkpoint inhibition is similar, reaching up to US$12,500 per month. These costs are due in part to the lack of laboratories that produce the therapies and a lack of standard production procedures which leads to variable outcomes and costs. Broadening the set of laboratories capable of producing immunotherapies as well as standardising the production methodology will be crucial to the widespread and accessible implementation of immunotherapy.

Despite these areas in need of improvement, immunotherapy remains an exciting and promising field of medicine. Novel types of immunotherapy come to surface each year, offering hope to many cancer patients.

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CHAPTER 7

Clinical applications of gene editing and therapy

7

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Introduction

The development of targeted gene therapies for patients with genetic disorders has been a goal for medical researchers for nearly five decades (Friedmann and Roblin 1972). Haematological diseases have been at the forefront of this endeavour and genetic therapies are rapidly becoming available as an important treatment modality for many haematological disorders.

Gene therapy in its broadest definition involves the introduction of exogenous genetic material into cells. This is usually but not exclusively achieved using viral vectors or nanoparticles. Cells can be modified with gene therapy approaches either *ex-vivo* by taking cells out of the patient, manipulating them and transplanting them back in, or *in-vivo* by directly introducing the genetic cargo to the target organs.

Genome editing is a relatively new technology that involves specifically modifying the sequence of the host genome to treat disease by precisely altering individual bases or disrupting particular sequences in their native genomic location. In some ways, this is distinct from conventional gene therapy, in which exogenous genetic material is added to cells, but there is considerable overlap because genome editing can also be used to insert sequences into the genome. The first cell therapy product based on genome editing technology obtained regulatory approval for the treatment of thalassaemia and sickle cell in December 2023 and this is likely to become an extremely important treatment modality in the future.

There are many potential applications for gene therapy and genome editing in haematology (Figure 7.1). Many are directed at correcting inherited genetic diseases such as haemoglobinopathies, immunodeficiencies and haemophilias. The other major application is to use exogenous genetic material to reprogram cells to treat disease. This is exemplified by the targeting of immune cells with gene therapy constructs to introduce chimeric antigen receptors, which target these cells to kill malignant cells. A third, important use of gene therapy approaches is to introduce genetic material to create an immune response in the context of vaccination.

In this chapter, we will largely focus on the principles behind genetic therapies and illustrate these using the paradigms of gene therapy and genome editing to treat inherited haematological disorders.

General principles of gene therapy

As early as 1961, it was shown that it was possible to make normal haemoglobin by adding free DNA to cultured cells from bone marrow taken from a patient with sickle cell disease (SCD). This was at levels well below that required for a clinical effect but established that in principle it is possible to correct genetic disorders by introducing exogenous DNA. In the 1980s, it was realised that genetic material could be much more efficiently delivered into haemopoietic cells, which undergo many rounds of cell division, using viruses that integrate into the host genome. Over the last 30 years several different types of viral vectors,

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

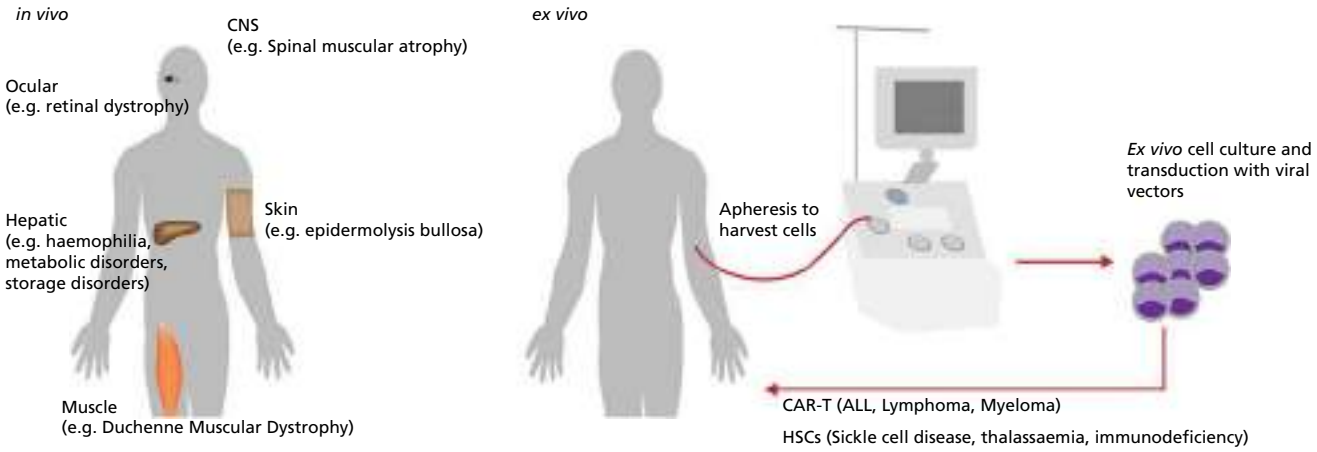

Figure 7.1 General principles of gene therapy and genome editing.

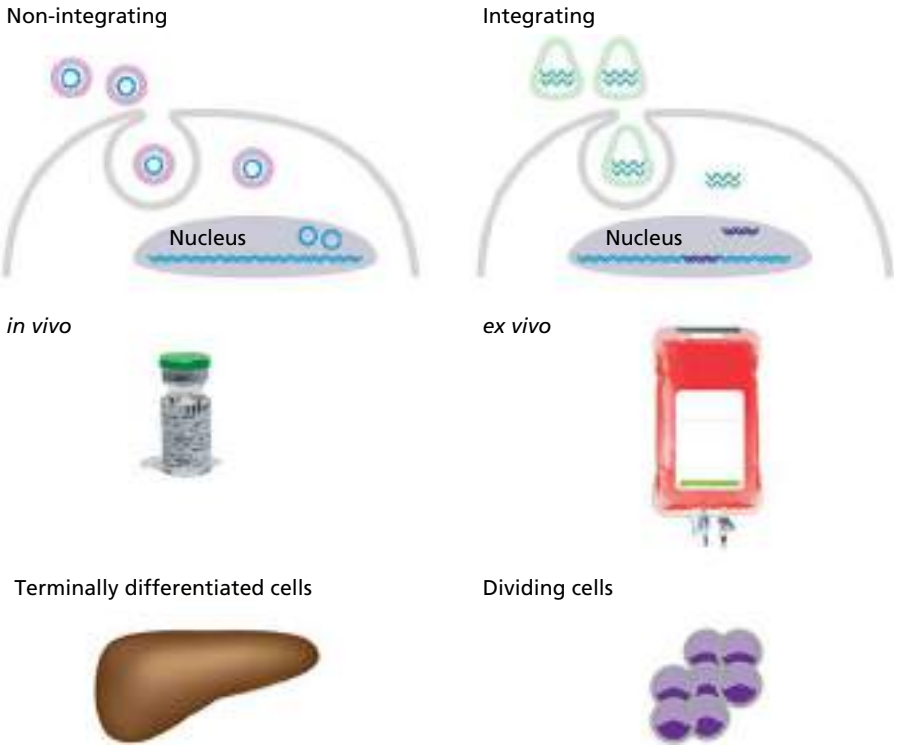

Figure 7.2 Integrating versus non-integrating viral vectors.

vehicles for delivering genetic material derived from viruses, have been used for gene therapy. These broadly fall into two categories depending on whether the DNA becomes integrated into the genome or not (Figure 7.2).

Vectors based on retroviruses (lentivirus, γ-retrovirus) integrate the viral genome into the host genome. This results in long-term retention of the genetic material from the vector because the DNA is replicated every time that the cell divides and all progeny therefore contain the viral construct. However, the viral vector integrates into the host genome in a random or semi-random manner. This can lead to disruption of genes or alterations in the way that genes within the host are controlled, which has the potential to cause malignant transformation.

Non-integrating viral vectors based on adenoviruses or adeno-associated viruses (AAV) deliver genetic material to cells effectively and only rarely integrate into the host genome. The risk of insertional mutagenesis is therefore much lower. However, over time the genetic material has the potential to be lost, particularly

from dividing cells. This is because the episomal viral genome is not replicated along with the host DNA during cell division and is therefore progressively diluted. Therefore, non-integrating viral vectors are only transiently effective in cells with rapid turnover, but they can generate effective levels of expression in terminally differentiated cell types. The age of the patient can make a significant difference to this effect, and it may prevent the use of non-integrating vectors in paediatric patients due to higher levels of cell proliferation compared to adults.

The vectors used for gene therapy have a differential ability to infect different cell types but they do not have perfect cell type specificity. For *ex-vivo* editing, this is not a significant problem because the target cell populations can be purified. However, for *in-vivo* gene therapy approaches, this is extremely important and there are limited numbers of cell types for which there are specific viral vectors available. Some degree of tissue specificity can be introduced into gene therapy approaches by engineering constructs with regulatory elements that encourage gene expression in that particular cell type. However, it is difficult to mimic the endogenous regulation of many genes accurately with a viral construct because many genes are controlled by multiple regulatory elements that act over large distances in the genome. This can be a problem when high levels of gene expression are required. Gene therapy approaches for haemoglobinopathies are particularly challenging because the gene requires expression at extremely high levels. This is an inherent limitation of gene therapy approaches compared to genome editing in which the endogenous gene can be repaired in its native locus with all its regulatory elements nearby.

Another important factor is that many viruses have a limited cargo size. AAV vectors in particular are unable to carry large genes as the wild-type genome is 4.7 kb in size, and this hampers gene therapy approaches for several indications (Wang *et al*. 2019).

Immunogenicity is a key consideration for *in-vivo* viral gene therapy approaches. Since many of the viral vectors used are naturally occurring, prior exposure to the virus or another closely related subtype can commonly occur and if this leads to an immune response with neutralising antibodies it will potentially lead to substantial reduction in the efficacy of treatment. The viral capsid proteins can be engineered to circumvent this problem, but immunogenicity will still be highly likely to prevent repeat dosing with the same therapeutic product.

Genetic material can also be packaged into synthetic nanoparticles which can be used to target entry into cells. There are a large variety of different approaches and this area is likely to expand very significantly in the future.

Gene therapy in haemopoietic stem cells

Although the earliest haemopoietic stem and progenitor cells (HSPCs) are generally quiescent, haemopoietic progenitors and precursors have extremely high rates of cell division and this means that integrating viruses are required for gene therapy approaches for immunodeficiency syndromes and haemoglobinopathies. As the haemoglobinopathies are encountered more frequently in haematology, we will focus on these in this chapter, but readers can be directed to reviews on gene therapy in immunodeficiency syndromes elsewhere (Ferrari *et al*. 2021; Houghton and Booth 2021).

Initially, Moloney leukaemia virus-derived γ-retroviral vectors were used in the first successful gene-therapy cure of an individual with a genetic disease; a 4-year-old girl with adenosine deaminase severe combined immunodeficiency (SCID). Whilst these approaches were efficacious, viral gene therapy has been beset by safety concerns. Notably, there were catastrophic outcomes in some early trials using γ-retroviruses, in which five patients with X-SCID and six with Wiskott-Aldrich Syndrome (WAS) developed T cell acute lymphoblastic leukaemia (T-ALL), with a further patient in the WAS trial developing acute myeloid leukaemia (AML). Furthermore, three of four patients with chronic granulomatous disease developed myelodysplastic syndrome following gene therapy. These transformations to leukaemia were found to be due to insertional mutagenesis at the regulatory elements of transcriptionally active oncogenes; integration into the host genome occurred in a non-random manner.

HIV-derived lentiviral vectors were developed to circumvent the problems associated with γ-retroviruses. Self-inactivating lentiviral vectors were developed with several important safety features. These included removal of the viral promoter from the U3 region of long-terminal repeats (LTRs) and modifications to the manufacturing process to prevent viral replication. Viral replication proteins were placed on different plasmids to those containing the genetic cargo for insertion. These steps have reduced the risk of transcriptional activation of nearby genes and viral-particle replication generating a safer integration profile. Most modern gene therapy approaches use self-inactivating lentiviral vectors.

Gene therapy for thalassaemia

The first individual with a haemoglobinopathy to be successfully treated with gene therapy had HbE/β-thalassaemia. The patient's cells were removed as in an autologous bone marrow harvest, transduced with a lentiviral vector known as HV569 containing a modified β-globin gene and reinfused after myeloablative chemotherapy. The modified version of β-globin involved an amino acid substitution at position 87 (T87Q), making it easy to track the globin produced by the gene therapy product compared to that produced by untreated cells. The patient was transfusion independent at 33 months (Cavazzana-Calvo *et al*. 2010). HbA^{T87Q} made up approximately a third of total haemoglobin in the patient at this time, and the levels stayed similar over the following decade of follow-up, with only occasional transfusions required.

Since then, more than ten phase I and II and two phase III trials have been conducted using lentiviral vectors to treat β-thalassaemia

and SCD using a gene addition or a shRNA knockdown of *BCL11a* approach (Ferrari *et al.* 2021). The BB305 vector (also known as Betibeglogene Autotemcel) was based on HV569, containing HBA^{T87Q}, and was used in phase I/II and III trials involving patients in the US, France, Thailand and Australia who had $\beta^0\beta^0$ and non-$\beta^0\beta^0$ genotypes. In the HBG-04 and HBG-05 studies, patients with a variety of genotypes received an infusion of modified autologous cells (Thompson *et al.* 2018). At a median of 26 months, follow-up 12 of 13 patients with a non-$\beta^0\beta^0$ genotype were transfusion independent, and of the 9 patients with $\beta^0\beta^0$ genotypes, the annualised transfusion requirement went down by 73%. However, only three of these patients became transfusion independent. A longer term follow-up of 4.9 years of patients on the HBG-05 study with transfusion dependent non-$\beta^0\beta^0$ thalassaemia found that all four patients remained transfusion independent. In the HBG-07 phase III study involving patients with non-$\beta^0\beta^0$ thalassaemia, 20 of 23 patients treated were transfusion independent, with a median follow-up of 29.7 months. 12 months after treatment the amount of gene-therapy-derived HBA^{T87Q} haemoglobin was 87 g/L, an improvement from the initial study in 2010. Results from the phase III HBG-212 study of patients with $\beta^0\beta^0$, $\beta^0/\beta^{+IVS\text{-}I\ 110}$, and $\beta^{+IVS\text{-}I\text{-}110}/\beta+^{IVS\text{-}I\ 110}$ genotypes are awaited.

An Italian phase I study utilised an alternative lentiviral vector, named GLOBE. Three adults and six children with $\beta^0\beta^0$ or severe non-$\beta^0\beta^0$ genotypes were treated with the vector. In contrast to the BB305 studies, the stem cell product was delivered intrabone rather than intravenously. At 24 months post-gene therapy all three adults showed a reduction in transfusion requirements, and three of four children achieved transfusion independence.

Finally, a US group utilised the TNS9.3.55 lentiviral vector in their phase I clinical trial. In contrast to the majority of other trials for haemoglobinopathies, reduced intensity conditioning was used prior to product infusion. At 6–8 years follow-up, the longest of the studies discussed here, none of the four patients were transfusion independent. This was felt to be due to the low vector copy number (the number of insertions of the genetic cargo per cell) in the product, 0.15. This was lower than the lowest vector copy numbers found in transfusion independent patients in the GLOBE and HBG trials (0.8 and 0.3 respectively).

Gene therapy for sickle cell disease

The vector used in the HBG trials, BB305 also used HBA^{T87Q}, which in addition to facilitating tracking conferred the β-globin chains with anti-sickling properties as it incorporated the amino acid in the γ-globin chain that is responsible for much of its anti-sickling properties, making the vector suitable for use in gene therapy for SCD. A single patient in France with $\beta^s\beta^s$ was treated with the BB305 vector as part of the HGB-205 trial. At 15 months follow-up approximately 50% of the patient's haemoglobin was comprised of the anti-sickling globin, which is consistent with levels of non-sickle haemoglobin found in patients with asymptomatic sickle-cell trait. There was no recurrence of sickle pain crises, an improvement in iron loading and he was transfusion independent. Longer term follow-up of the HGB-205 trial involved this patient (SCD-1) and two other patients (SCD-2 and SCD–3). The results were mixed, with SCD-2 continuing to suffer pain crises and acute chest syndromes with recommencement of hydroxyurea and exchange transfusions. SCD-1 has had one pain crisis to date since the therapy, but otherwise, he and SCD-3 have remained complication-free and are transfusion independent at 6.7 and 4.3 years post-gene therapy. Subsequent modifications to the protocol included peripheral blood stem cell collection with plerixafor mobilisation to improve the cell dose obtained and an improved manufacturing and transduction process. Results of the phase I/II HGB-206 study were recently published, including 35 patients with median follow-up of 17.3 months (Kanter *et al.* 2022). The mean haemoglobin concentration increased from 85 to 110 g/L or more, with HbA^{T87Q} contributing at least 40% of total haemoglobin. Of 25 patients who were available for evaluation, all had complete resolution of severe vaso-occlusive events. Following this trial lovotibeglogene autotemcel was approved by the FDA for the treatment of patients over the age of 12 with veno-occlusive events.

An orthologous approach has been to use a lentiviral vector to deliver a version of the γ-globin gene instead of β-globin, in order to harness HbF's potent anti-sickling activity. A phase I/II trial using the vector ARU-1801 which contains the modified gamma-globin gene γ^{G16D} delivered using a reduced-intensity conditioning regimen is currently ongoing with promising results (Grimley *et al.* 2021).

Finally, rather than deliver a form of β- or γ-globin, another approach has been to reduce the erythroid-specific expression of the transcription factor BCL11A, which increases γ-globin expression (explained further below). A microRNA-adapted short-hairpin RNA was delivered by a lentiviral vector, which post-transcriptionally silences *BCL11A* expression in erythroid cells through RNA interference. Six patients were initially treated at Boston Childrens' hospital with a median follow-up of 18 months. HbF-induction ranged between 20.4 and 41.3%, and no patient has had an acute pain crisis, chest syndrome or stroke since receiving gene therapy, with two of three patients who were transfusion dependent pre-gene therapy no longer needing red cells.

Safety and efficacy concerns

Given the previous malignant transformations in patients treated with γ-retroviral vectors, patients enrolled on lentiviral vector trials have been monitored closely. In the initial lentiviral trial, approximately 45% of vector-bearing nucleated red blood cells were found to contain a clonally expanded insertion of the

construct within the third intron of the gene *HMGA2*. To date, these clones have not progressed into a malignant disorder. More concerningly, 5.5 years after treatment, a female with SCD in HBG-206 Group A developed AML (Goyal *et al*. 2022). 86.7% of CD34+ bone marrow cells at AML diagnosis were lentiviral vector positive, and the relative frequency of insertions at a particular gene, *VAMP4*, was 98.6% at this time. However, as there was low transgene expression in blast cells, no perturbation of *VAMP4* expression or that of surrounding genes and the presence of known molecular and cytogenetic AML-driver mutations, it was felt that the AML was unlikely to be related to the vector insertion. A similar conclusion was reached in the case of another patient with SCD in HBG-206 Group A who developed MDS and then AML, though in this case vector insertion in blast cells was not detected. Notably, SCD is known to be a chronic inflammatory disorder, with an excess risk of developing malignant haematologic disease. Clearly longer term follow-up is needed to fully assess the safety profile of patients with haemoglobinopathies receiving viral-vector-based gene therapy.

Gene therapy for haemophilia

Gene therapy is becoming an important treatment modality for haemophilia and the first gene therapy products have recently received authorisation by regulatory agencies in the UK, Europe and the United States. Here *in-vivo* gene therapy approaches have been used with non-integrating viruses. Hepatocytes are readily infected by non-integrating AAV vectors and because these cells do not undergo rapid turnover, in contrast to HPSCs, persistent expression from AAV vector constructs has been seen several years after treatment. In children, hepatocyte proliferation is greater than adults and it is likely that gene therapy in children will lead to gradual loss in factor production as they progress into adulthood.

Immunogenicity is a major concern with current AAV vector gene therapy approaches. Around 30–80% of the population have neutralising antibodies to AAVs depending on the serotype of the virus and the geographical location of the study. Generally, the presence of neutralising antibodies is an exclusion criterion for treatment at present. Following administration of AAV vectors there is a very potent immune response probably making repeat dosing ineffective.

AAV vector approaches have been developed for both Factor VIII (FVIII) and Factor IX (FIX). Development of gene therapy for haemophilia B has been more straightforward than for haemophilia A for a number of reasons. First, the gene is smaller and can easily be packaged into AAV vectors, whereas the FVIII gene is too large (cDNA ~9 kb) and so all current transgene approaches have used truncated forms of the gene, generally with the B domain removed. Secondly, FIX is synthesised natively by hepatocytes whereas FVIII is largely synthesised in endothelial cells. Finally, there is a gain of function variant for FIX (FIX Padua), which allows therapeutic FIX activity with lower protein levels.

Therefore, the first successful gene therapy trials were for haemophilia B but in the last few years gene therapy for FVIII has caught up and fully licenced products became available for haemophilia B (etranacogene dezaparvovec) in late 2022 and haemophilia A (valoctocogene roxaparvovec) in 2023.

The main side effect of AAV gene therapy approaches for haemophilia is hepatic toxicity, which usually occurs 1–3 months following treatment. The pathogenesis is not completely understood but it likely relates in part to the immune response to the AAV vector and partly due to direct hepatocyte toxicity caused by high levels of clotting factor protein expression, which is a particular problem with FVIII therapy. This is treated empirically with corticosteroids (e.g. prednisolone 1 mg/kg) with addition of other immunosuppressants in a subgroup of patients who fail to respond.

The longer term safety of gene therapy for haemophilia will not fully be known for several decades. Long-term follow-up of these patients is important and the international haemophilia gene therapy registry is being developed to facilitate vigilance.

The duration of responses following AAV therapy can be excellent. From the initial haemophilia B study, only a minimal decline in FIX levels was seen at 8 years post-treatment. However, this may not hold true for all cases. In the longest duration FVIII study, levels have been seen to decline substantially at 4 years post-treatment down to 0.2 IU/mL; a level which provides substantial bleed protection but it is unclear whether this will continue to fall. Predicting the clotting factor levels post-therapy has proven to be difficult. In human trials to date, there has been a 10-fold range in variability between different subjects (0.2 to >2.0 IU/mL). This variability likely results from a number of factors including how well the virus enters hepatocytes, the process by which the transgene remains episomal, the complexity of production of the protein by hepatocytes and differences in secretion and clearance of the proteins, which is also highly variable for FVIII and FIX in the normal population.

Genome editing

Within the last decade, huge advances have been made in genome editing technologies. These allow the genome of human cells to be altered precisely. This is the beginning of a new era for us as a species because we are now not only able to read our own genetic code but also edit it at will. It is worthwhile thinking about the scale of what this technology allows us to do. The human genome is made up of 3 billion base pairs and we are now able to change the sequence of the genome precisely in millions of cells simultaneously at nearly any site we choose, with high efficiency. It is an incredible feat which will almost certainly lead to huge advances in therapeutics and not simply for inherited genetic disorders, whilst at the same time raising

important ethical concerns. Regulatory approval for the first genome edited product for treatment of thalassaemia and SCD (exagamglogene autotemcel) was obtained in late 2023.

Genome editing relies on the use of proteins known as endonucleases, which can be programmed to bind to specific DNA sequences. These endonucleases have an intrinsic ability to cut DNA at their binding site but they can be attached to other proteins to confer a variety of different effects at the target site. Several different types of programmable nucleases have been developed including meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) but the discovery that the clustered regularly interspersed short palindromic repeat (CRISPR)/CRISPR associated proteins (Cas) system could be used for genome engineering transformed the field (Kim and Kim 2014).

The CRISPR/Cas system evolved in bacteria as a defence against infection by viruses. First described in 1993, it was found to utilise *cas* endonucleases that could cut and neutralise viral phage DNA but only when the virus had previously been encountered. We now know that during viral infection in bacteria, part of the viral DNA becomes incorporated into the CRISPR sequences in the bacterial genome and this directs the *cas* proteins to cut and neutralise that sequence in subsequent infections. In 2012, two groups used recombinant Cas9 and *in-vitro* transcribed RNA to show that the CRISPR/Cas9 system could be reprogrammed to cut DNA *in-vitro*. This RNA, known as a guide RNA (sgRNA) is complementary to a target genomic sequence and guides the Cas9 endonuclease to theoretically any sequence in the genome. This is hugely powerful because it can be used to cut any DNA sequence and this work earned Jennifer Doudna and Emmanuelle Charpentier the Nobel prize for chemistry in 2020. The potential bioengineering and therapeutic applications were very rapidly exploited and the following year the system was adapted for use in mammalian cells.

General principles of genome editing

Several different types of genome editing have been developed (Figure 7.3). Initial approaches used endonucleases to create a double-stranded break (DSB) in the DNA at the target site. This is usually repaired by the non-homologous end joining (NHEJ) pathway, which usually causes a small deletion at the target site although occasionally insertion of small sequences can occur. This process can have very high editing efficiencies (even as high as >95% of alleles) and several approaches have been developed to utilise these small deletions for therapeutic effect but generally, it is not ideal for repairing mutations precisely because a mixture of different editing outcomes are seen leaving a 'scar' at the target site.

Alternatively, the homology-directed repair (HDR) pathway can seamlessly repair the DSB and this can be used to change the

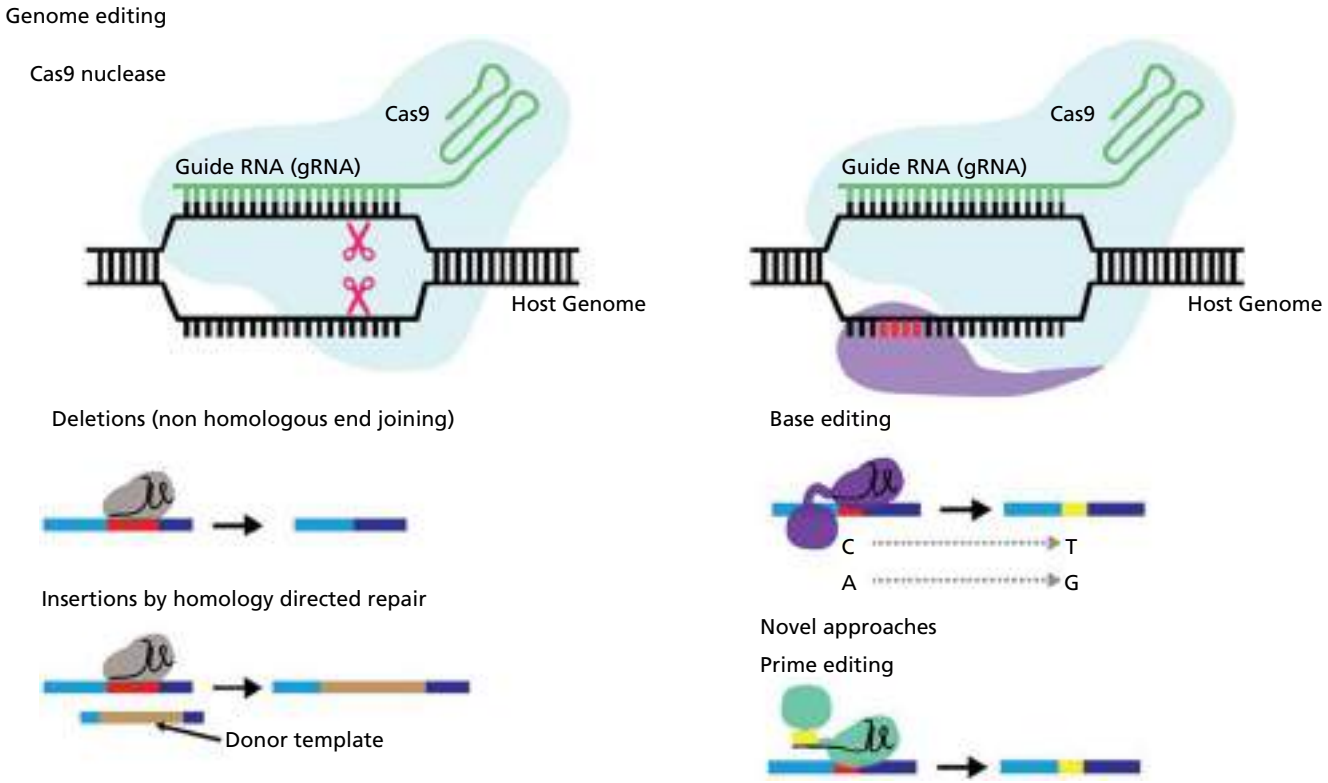

Figure 7.3 General principles of genome editing.

DNA sequence by supplying a DNA template with the desired repair sequence. Whilst this can potentially repair genetic mutations, such as the SCD mutation, the process is not as efficient as NHEJ. Furthermore, HDR preferentially occurs in actively dividing cells and quiescent HSPCs are therefore more challenging to edit with HDR.

A third alternative is the use of base editors. Base editors are naturally occurring deaminases that are able to irreversibly change one DNA base to another *in-situ* without the need for DSBs.

These systems allow transitions of A–G and C–T but not transversions from purine to pyrimidine or vice-versa (A–C or G–T). Recently glycosylase base editors have been reported which allow conversion of purine to pyrimidine but these have lower editing efficiencies. Base editors have the potential to make highly specific edits and the off-target effects may be lower than with conventional genome editing.

Further experimental approaches have been developed that further extend the scope of genome editing. For example, prime editing uses a reverse transcriptase to convert part of the guide RNA into a template to alter the DNA sequence. This has the potential to allow highly efficient repair of small deletions or sequence changes that cannot be achieved with base editors. Finally, another area with therapeutic potential is termed epigenome editing. Here the genetic code is left unchanged but the epigenetic annotation of the DNA and histones is altered and this can be used to both turn on genes and inactivate them. This can result in changes in gene expression that persist despite many rounds of cell division.

Delivery of genome editing technology

In contrast to conventional gene therapy, the editing proteins and RNAs only need to be expressed by cells for a short period of time because once the DNA sequence has been edited it will remain altered indefinitely in the cell and its progeny. Editing machinery can be delivered into the cell in one of three broad ways: electroporation, lipofection and viral transduction (Stewart *et al*. 2016).

Electroporation is a membrane-disruption-based approach first used in 1982. Cells and the material to be electroporated are held in suspension, between two electrodes and electrical pulses are used to transiently disrupt the membrane. Whilst there is some cytotoxicity, partly depending on the biomolecules being transferred, electroporation is commonly used as it is rapid, is applicable to a diverse set of primary cells, has a defined period of transfection, is straightforward to perform and is not immunogenic. For human clinical uses, electroporation is only used *ex-vivo*.

As with gene therapy, gene editing components can be delivered into the cell via viral vectors, with similar strengths and weaknesses as described above. Non-viral carrier-based techniques involve the use of lipoparticles, polymers or other non-organic nanoparticles to enclose editing machinery and deliver it to the cell through fusion with the cell membrane. These techniques are attractive as they potentially circumvent cellular toxicity of electroporation and the genotoxicity of viruses. In addition, they could lend themselves to *in-vivo* therapy.

CRISPR/Cas9 components are delivered into the cell in several different forms: as DNA, mRNA or pre-formed ribonucleoproteins (RNPs). If DNA is delivered, the cell's machinery must transcribe the DNA into mRNA and translate this into Cas9 protein before editing can occur. Nucleic acid and viruses often induce type I interferon responses in the host cells. Alternatively, the editing machinery can be delivered as mRNA. This results in more rapid, transient editing because it only requires the protein to be translated by the host cells. Finally, very high editing efficiencies can be achieved by manufacturing Cas9 protein, incubating it with the sgRNA and delivering this as an RNP. Current clinical trials using CRISPR/Cas9 for patients with SCD and β-thalassaemia have used RNPs combined with *ex-vivo* electroporation of CD34+ HSPCs. However, *in-vivo* approaches with viruses are beginning to come into the clinic for other indications.

Genome editing for the β-thalassaemias and sickle cell disease

The β-haemoglobinopathies are an attractive candidate for the use of CRISPR/Cas9-based gene editing. Decades of research means that the α- and β-globin loci are amongst the most well studied and best-understood genes in the genome in terms of their regulation. In addition, the technology for harvesting and transplanting HSPC has been optimised over the last 50 years and it is well understood how to perform haemopoietic stem cell transplantation for patients with haemoglobinopathies. This allows *ex-vivo* editing to be used to correct these defects. Combined with the huge burden of disease this has made correction of haemoglobinopathies one of the first targets to enter the clinic and multiple approaches have been developed, which demonstrate many of the key principles of genome editing (Figure 7.4).

Approaches for treating haemoglobinopathies through elevation of fetal haemoglobin levels

One of the tragedies in SCD and β-thalassaemia is that the vast majority of patients have a completely normal fetal haemoglobin gene, and they only develop disease because this gene becomes repressed during the first few months of life. Understanding this switching process has been the focus of huge research effort. A key advance came in 2007 when Swee Lay Thein's group at King's College London discovered that the *BCL11A* gene plays a critical role in determining HbF levels (Menzel *et al*. 2007). *BCL11A* has subsequently been shown to be a developmental-stage specific repressor of γ-globin expression and it can be targeted for

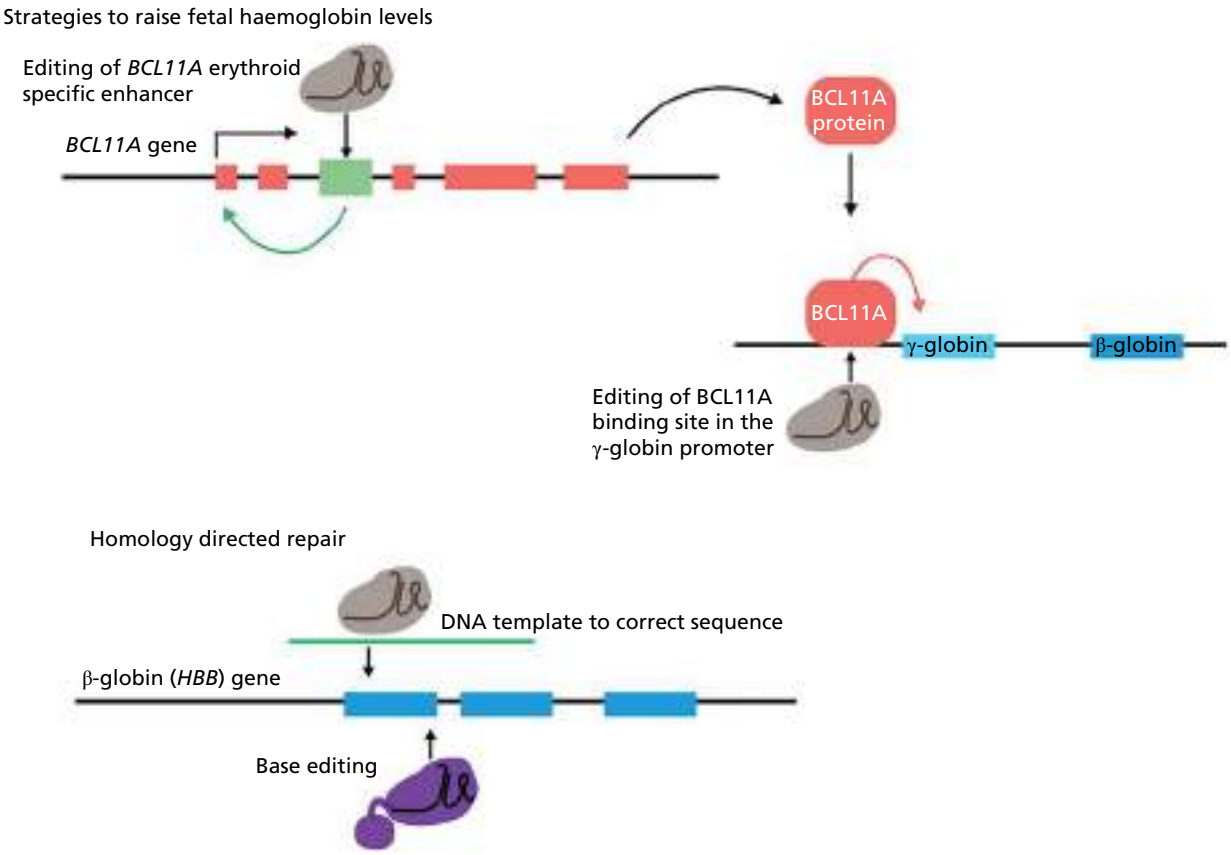

Figure 7.4 Different approaches for using genome editing to treat haemoglobinopathies.

therapeutic reactivation of HbF. A significant advantage of this approach is that it can be used to treat nearly all β-haemoglobinopathies irrespective of the mutation.

Reducing expression of *BCL11A* results in increased HbF levels but the gene is also important in lymphoid development so targeting of the gene itself is not possible without causing immunodeficiency. However, the expression of *BCL11A* can be reduced specifically in erythroid cells through editing of the sequences that control when the gene is turned on (known as enhancers). *BCL11A* and other repressors are known to bind to the promoter of the *γ-globin* gene. These binding sites are known to occur in hereditary mutations causing hereditary persistence of fetal haemoglobin (HPFH).

The first phase 3 studies in patients with β-thalassaemia and SCD using the *BCL11A* enhancer-editing approach have been undertaken and are promising (Frangoul *et al.* 2024; Locatelli *et al.* 2024). It is possible to manufacture edited CD34+ cell products with editing efficiencies of >70%. 32 of 35 patients treated for β-thalassaemia became independent of blood transfusion with a mean Hb level of 131 g/L, with a median follow up of 20.4 months post-transplant. For SCD HbF levels of 43.9% were achieved and 29 out of 30 patients with sufficient follow up were free from vaso-occlusive crises for at least 12 consecutive months although 6 of 43 patients had a crisis at some point post transplant. These patients had significant adverse events because the approach requires full-intensity conditioning. The most serious events were associated with prolonged neutropenia (median neutrophil engraftment occurred on day +27 (range 15-40)). The long-term safety of these approaches is likely to be favourable compared to gene therapy approaches, but the full safety will not be known for many years.

Approaches for treating haemoglobinopathies through direct correction of the gene

It is possible to repair mutations using a repair template through homology-directed repair (HDR) pathway. Unfortunately, HDR is less efficient than NHEJ and HDR preferentially occurs during cell division and this particularly makes it challenging to edit haematopoietic stem cells, which are largely quiescent.

Despite these problems, HDR approaches have been optimised in HSPCs, and reasonably high editing efficiencies are possible. In particular, an approach which combines Cas9 in ribonuclear protein format with an AAV viral vector to deliver

the HDR template can achieve editing efficiencies in the order of 60%. These approaches are likely to enter into clinical trials in the near future.

Base editors, which directly modify the DNA at the target site can also be used to correct mutations but with significantly higher editing efficiencies than HDR. The potential disadvantage of this approach is that a specific editing strategy has to be developed for every mutation and these enzymes are only able to convert C–T or A–G, meaning that not all variants can be corrected. However, the two most important variants of β-globin in terms of disease burden (HbS and HbE) can be corrected with base editors. Interestingly HbS is an A to T transversion which is difficult to correct with a base editor, however, it can be corrected to a non-pathogenic variant (Hb Makassar), which does not cause a sickling phenotype. Similarly, HbE can be corrected to a variant haemoglobin (Hb Aubenas), which also has a normal phenotype.

Safety of genome editing

Whilst genome editing offers an attractive alternative to random/semi-random viral integration-based therapies, there are still safety concerns surrounding adverse editing outcomes. Characterisation of these events is complex because mutations and other forms of cellular toxicity can occur at any point during the manufacturing process. In addition, HSPCs from patients may carry higher rates of underlying mutations and pathology may only occur after additional mutations accrue *in-vivo* after cells have been transplanted, which means that it will take many years for the toxicities of cellular therapies to be fully known. However, it is important to weigh the potential toxicity against the toxicity of the underlying disease process and the toxicity of other treatment options. For many conditions which severely limit life expectancy and quality of life, this means that substantial levels of off-target effects are acceptable but clearly patients will need to be counselled carefully until the safety is fully known.

Unintended consequences from genome editing can occur at the site of editing. When an editor is used to make a deletion the majority of editing outcomes will be mediated by NHEJ and most of these outcomes are deletions that are under 50 bp. However, it has been observed that large deletions spanning many kilobases can also be generated. These have even been shown to extend to megabase scale, and involve chromosomal losses in human embryos.

Cells can develop chromosomal translocations at the editing sites, particularly if more than one site is being targeted simultaneously. Providing oncogenes are avoided, these are unlikely to be pathogenic but deliberate cutting of the genome at sites associated with recurrent translocations in haematological malignancy will cause pathogenic translocations. Beyond simple deletions and translocations, more complex local and large-scale rearrangements have been observed, ranging from small inversions to chromothripsis (chromosome shattering). DSB formation following Cas9 editing has been shown to activate the cellular p53 DNA damage response, with potential to confer a selective advantage to cells carrying a *p53* mutation.

A second major adverse consequence of editing is 'off-target effects'; editing at sites that have sequence similarity to the guide RNA directing the editor to the target site. This off-target editing could potentially cause disruption to genes and sequences anywhere in the genome. Potential off-target sites can be profiled with computational and molecular biology approaches and targeted sequencing approaches allow rare mutations at these sites to be detected. It is possible to profile the consequences of these types of off-target effects and redesign unfavourable targeting guide RNAs. Thus far trials using conventional Cas9 off-target events have not been problematic, with the caveat that current sequencing approaches do not have sufficient sensitivity to readily detect sporadically occurring mutations in populations of cells.

Conclusion

Haematological diseases and the manipulation of haematopoietic cells have been at the forefront of genetic therapies, from the inception of the field in the 1970s and 1980s. Recent technological advances have seen a rapid expansion in this area in recent years, with high-profile trials yielding very promising results. With the development of CRISPR/Cas9-based strategies the promise of meaningful long-term cures for many inherited blood disorders is closer than ever. However, the permanent and irrevocable nature of these treatments, combined with the lack of long-term safety data and the rapid pace of developments both in genetic therapies but also in alternative conventional therapeutics, presents a major challenge for clinicians and patients. The decision whether to go ahead with a therapy that will last the duration of the patient's lifetime and is likely to be superseded by safer alternatives will have to be carefully balanced with the patient's symptoms and prognosis. Given the uncertainties and the lack of a straightforward approach to patient selection, these patients will need to be carefully advised prior to treatment. Translating these early successes into accessible and affordable therapeutics for patients with both common and rare disease will be a major challenge for the field. However, it is likely that gene editing will become a major treatment avenue for patients with haematological disease in the decades to come.

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CHAPTER 8

Application of next-generation sequencing in haematology

8

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Introduction

Since the discovery of the Philadelphia chromosome in chronic myeloid leukaemia and the *HBB* p.(Glu6Val) amino acid substitution in sickle cell disease, it has been clear that genomic investigation has a key role to play in the diagnosis and management of a broad range of both malignant and non-malignant haematological disorders. Although the use of genomic techniques in haematology is not new, over the last decade there has been widespread adoption of Next-Generation Sequencing [NGS] technologies allowing the rapid acquisition of much larger quantities of genomic data than previously achievable.

NGS, also referred to as massively parallel sequencing, relates to technologies which permit multiple nucleic acid fragments to be sequenced simultaneously, allowing many regions from numerous individuals' genomes to be analysed in a single reaction. An NGS assay can vary from sequencing a few hundred bases of an individual's genome to the entire genome (Whole-Genome Sequencing [WGS]). In addition to the speed and data breadth advantages this brings over conventional Sanger sequencing, the ability to analyse multiple fragments of nucleic acid covering any single region of the genome means the depth of sequencing and, therefore, sensitivity is much higher. Consequently, mutations with lower allele frequency changes can be detected, which is often highly relevant in the acquired disease setting. The massively parallel nature of the technologies also significantly reduces the cost of sequencing improving its affordability for routine implementation in appropriate clinical scenarios.

NGS technology

A summary of some of the technologies underpinning NGS is shown in Figure 8.1 and discussed in greater detail below. These can be divided into short-read technologies which typically sequence 50–300 base pairs [bp] in each read (nucleic acid fragment) and long-read technologies which can sequence over 10,000 bp (i.e. 10 kb) in a single read. Currently, short-read technologies constitute the bulk of clinical NGS services but long-read technologies are being explored for possible implementation. Whichever technology is used, there are some pre-sequencing steps which are common to most and are encompassed in the process of 'library preparation', with the library being what will ultimately be sequenced. Differences in library preparation relate to the nature of the input substrate (i.e. DNA, RNA or cDNA), the scope of the assay (breadth of genome to be sequenced and variant type to be detected), and in the case of a targeted panel whether amplicon or hybridization technology is being employed.

Choice of input substrate

The choice of assay substrate will depend on the combination of the type of genomic information required and the technology being used. Typically, with short-read technologies DNA is used

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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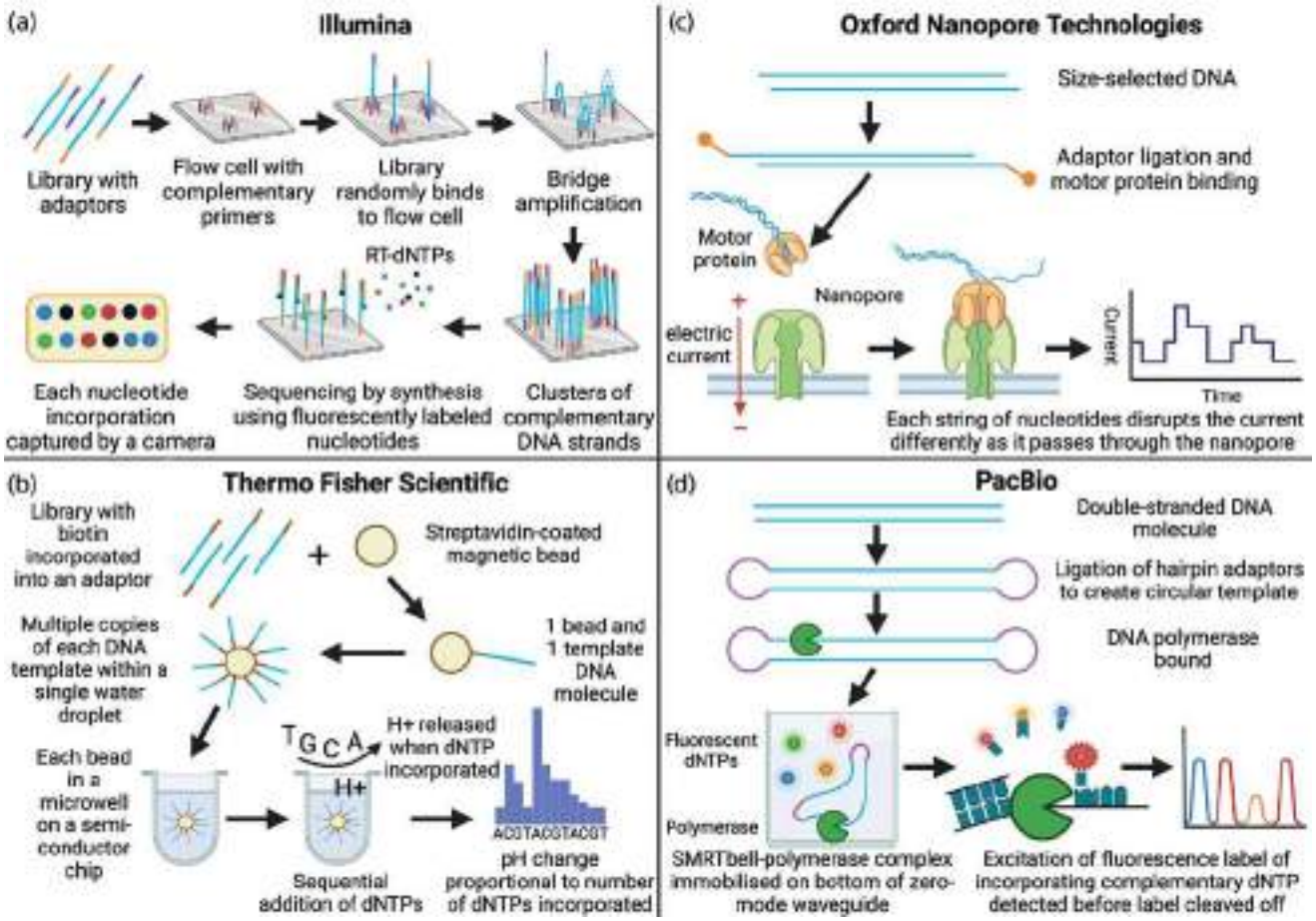

Figure 8.1 Outline of the workflow for commonly used short and long-read sequencing technologies. (a) Illumina, (b) Thermo fisher scientific, (c) Oxford Nanopore Technologies, (d) PacBio. Created with BioRender.com.

for small variants (often classified as single nucleotide variants [SNV] and small insertions or deletions up to 50 bp) and copy number variant (CNV) detection, while cDNA reverse transcribed from RNA is used to identify structural variants (SV) (i.e. inversions and balanced translocations) and measure gene expression (i.e. to what degree a gene is being transcribed to RNA allowing its protein product to be produced). However, it is possible to detect structural variants using DNA, particularly if extensive intronic and intergenic regions are sequenced in the assay. Where a translocation brings a promoter/enhancer from one gene in proximity to the coding region of another gene (e.g. *IGH* translocations with *MYC*, *CCND1* and others), it is preferable to use DNA as the substrate as promoter/enhancer regions do not undergo mRNA transcription and therefore such translocations will not be detected using cDNA as the substrate.

The tissue of origin of the nucleic acid substrate (and its preservation) can also have implications for the choice of downstream assay. Typically, peripheral blood can be used for the detection of germline variants in non-malignant haematological disorders; this is usually a bountiful source of high-quality fresh DNA and RNA. Many haematological malignancies are present in either peripheral blood and/or bone marrow aspirate samples, similarly providing an excellent source of nucleic acids. Some haematological malignancies, however, are only found in tissue biopsies (often from lymph nodes) or bone marrow trephines both of which usually undergo formalin fixation followed by paraffin embedding (FFPE) in order to aid morphological assessment.

Formalin fixation can cause hydrolytic deamination of cytosine to uracil which can result in artefactual C:G>T:A substitutions upon sequencing. Extraction of nucleic acid from paraffin often involves the use of sufficient heat to cause significant fragmentation of the DNA resulting in very short molecules; this precludes nucleic acids obtained from FFPE material being used in long-read sequencing and can limit the ability of short-read sequencing to detect more complex genomic abnormalities from this sample type. An additional consideration with using bone marrow trephine samples as a source of nucleic acid is the decalcification method employed with EDTA (i.e. E hylenediaminetetraacetic acid – a weak acid) allowing

extraction of much higher quality DNA compared to more traditional decalcifying agents such as hydrochloric acid.

In certain circumstances, it is desirable to undertake testing of a germline sample in haematological malignancies: This may be to confirm that an abnormality detected in a tumour sample was constitutional in origin rather than somatic. Alternatively, it may be in the context of a broad assay like WGS where the availability of the germline sequence will allow, through a tumour:normal subtraction, the determination of which variants are acquired and therefore associated with the development of the malignancy. In the event, the malignancy is known or suspected to be in the peripheral blood this cannot be used as the germline source as the presence of tumour in the germline sample will result in the removal of the tumour variants at the time of the tumour:normal subtraction. Many alternative samples have been investigated as potential germline samples in haematological malignancies. Although saliva and uncultured skin samples are usually an adequate germline for lymphoid malignancies, they are often relatively heavily contaminated with myeloid lineage cells (usually neutrophils) which can harbour the 'tumour' mutations. DNA obtained from nails is often of poor quality and although usually uncontaminated; DNA from hair follicle cells can be limited in quantity. It can also be challenging to sort CD3+ cells to sufficient purity and in sufficient quantity for NSG assays with higher DNA input requirements and these may contain any mutations which arose at the level of the haematopoietic stem cell. Therefore, the gold standard germline sample is cultured fibroblasts although it is noted that this both involves the patient undergoing a skin biopsy and the requirement for this to undergo *in vitro* culture which may not be routinely available.

Choice of assay breadth and depth

The breadth of an assay or footprint (i.e. region of the genome to be sequenced) will depend on what genomic information is desired. In both malignant and non-malignant haematology, there is often a defined list of variants to be demonstrated or excluded which can be appropriately accommodated on a targeted panel. If information about CNVs is required, a single nucleotide polymorphism (SNP) backbone can be deployed across a DNA-based panel. Through the even distribution of these regions containing polymorphisms across the genome, deletions/amplifications and regions of loss of heterozygosity (LOH) (i.e. where one allele is lost either due to deletion or in the case of copy neutral LOH replacement with the other allele) can be inferred without the need to sequence the entire genome.

The depth of sequencing (i.e. number of reads covering a region of the genome) required for a DNA-based assay will depend on the expected variant allele frequency of the genomic abnormalities (i.e. percentage of analysed DNA at that position having the abnormality): In non-malignant haematology, the inherited i.e. germline variants under investigation would be expected to have allele frequencies of ~50% or ~100% (providing there is no germline mosaicism). Panels designed to detect only germline variants usually have a minimum coverage of ~30× (i.e. at least 30 sequencing reads cover each nucleotide in the target regions). In contrast, acquired variants would be expected to have highly variable allele frequencies depending on whether they are fully clonal or sub-clonal and the proportion of mutated cells in the blood, bone marrow or tissue. Most assays designed to test for acquired variants have a minimum coverage of ~250× (although many often sequence deeper) in order to be able to detect variants with an allele frequency of at least ~5–10%.

An alternative to a targeted panel, particularly if there is any ambiguity surrounding the diagnosis, is to perform Whole-Exome Sequencing (WES) and then apply virtual panels to the data bioinformatically. This DNA-based technique sequences all the protein-coding regions (i.e. exons) of the genes in a genome (~1% of the whole genome) so is ideal for detecting SNVs and CNVs involving exons. Target sequencing depth is typically 30–100× depending on its application. Although used by some in malignant haematology diagnosis, it will not detect many translocations owing to the absence of sequencing covering intronic and intergenic regions. It is, however, increasingly used in non-malignant haematology to identify germline variants and allows sequential panels of gene targets to be applied to data, particularly useful if a phenotype evolves or new data becomes available relating to causative mutations for any specific disease. This approach removes the need for laboratories to continually validate new targeted panels as genomic knowledge increases.

For an unbiased approach for SV detection or gene expression quantification, whole transcriptome analysis/whole RNA-seq can be used. The former analyses coding and non-coding RNAs while the latter concentrates on mRNA. Although such comprehensive RNA analyses are currently more typically employed for research purposes, whole transcriptomic analysis is being used by some in clinical practice in combination with either WES or WGS to provide the most comprehensive analysis of the genome. Aside from the additional gene expression data (which for a genome-wide analysis would normally require a microarray), when combined with WES it provides the 'missing' SV data, and when used in conjunction with WGS it provides information as to whether SVs at the DNA level result in altered mRNA expression, therefore suggesting they are functional.

WGS is a DNA-based assay which aims to sequence the entire genome and therefore can detect all variant types, including translocations. If being used for germline variant detection sequencing at a depth of ~30× is usually considered adequate whereas if discovery of acquired variants is required, depths of ~70–100× are usually aimed for.

Amplicon versus hybridization panel

Broadly, two different approaches exist for the enrichment step of library generation for targeted panels as shown in Figure 8.2. The amplicon-based approach first involves a multiplex

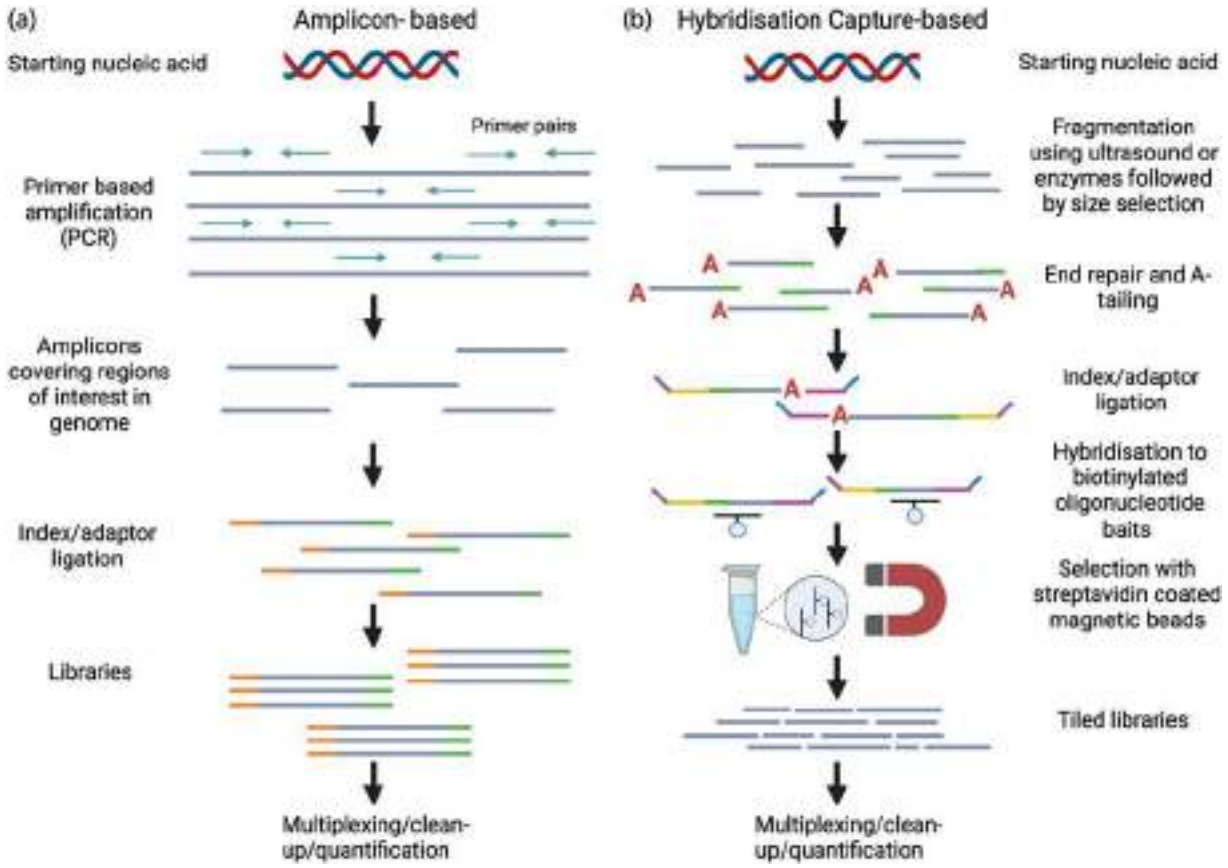

Figure 8.2 Outlines the main steps in Amplicon-based and Hybridization Capture-based library preparation. (a) amplicon-based, (b) hybridization capture-based. Created with BioRender.com.

Polymerase Chain Reaction (PCR) which uses oligonucleotides flanking the regions of interest in the genome, allowing their amplification. The hybridization capture-based approach involves first fragmenting the genome (most commonly performed ultrasonically or enzymatically), before incubating with biotinylated oligonucleotide probes which are complementary to regions of interest. Nucleic acid fragments hybridised to the probes are then selected using streptavidin-coated magnetic beads. Each approach has advantages and disadvantages as outlined in Table 8.1.

The fragmentation step of hybridization panels means nucleic acid molecules sequenced covering any particular target will have a large range of combinations of first and last nucleotides. Therefore, it is much easier to identify families of identical reads likely to have been generated from a single starting molecule which can then be collapsed (i.e. PCR repeats removed), allowing only one read in the family to contribute to the final sequencing result. In contrast, all reads derived from an amplicon-based approach covering any particular target will have the same first and last nucleotides as these have been determined by the oligonucleotide primers used. In this situation without the use of Unique Molecular Identifiers/Indexes ([UMIs] – described below), it is not possible to remove PCR duplicates meaning it is not possible to exclude a variant being a sequencing artefact.

Library preparation

Figure 8.2 outlines the specifics of library preparation for the two targeted panel approaches but there are additional steps which are common to both. In order that DNA from more than one individual can be sequenced in a single reaction (i.e. multiplexed), it is necessary to 'label' the DNA from each individual with an index (or indexes); these are short sequences of DNA which are ligated to one or both ends of the DNA molecules originating from an individual which ultimately allow each sequencing read to be assigned to the correct person's genome.

In addition to the ligation of indexes, various platform-specific adaptors are used, particularly in sequencing by synthesis methods which allow DNA molecules to adhere to surfaces (e.g. beads or flow cells) where sequencing steps are taking place. Other common steps include size selection/clean-up steps (where DNA molecules of inappropriate length and excess indexes and adaptors can be removed) and DNA quantification steps (can be fluorescence or amplification-based) to ensure

Table 8.1 Comparison of advantages and disadvantages of hybridsation-based versus amplicon-based library preparation.

	Hybridization-based library preparation	Amplicon-based library preparation
Workflow	More complex (typically 1–2 days)	Less complex (typically <1 day)
Cost	Varies*	Generally considered to be cheaper*
Input nucleic acid	>10 ng**	<10 ng**
Capture size	Virtually unlimited	Usually <10,000 amplicons
Assay characteristics	More even coverage of targets owing to less differential annealing of oligonucleotide probe More off-target reads owing to probes annealing to non-target regions	Less even coverage of targets owing to differential annealing of oligonucleotides leading to amplification bias Fewer off-target reads
Sensitivity	~1% with removal of PCR repeats without the need for UMIs	~5% in the absence of UMIs
Variant detection	SNVs/small indels CNVs	SNVs / small indels

* Cost will vary depending on the specific design of the assay but for smaller footprints amplicon panels are generally considered to be cheaper than hybridization panels overall.
** The required nucleic acid input will vary depending on the specific assay but amplicon panels generally can tolerate lower inputs than hybridization panels.

that when libraries from different individuals are combined the desired sequencing depth for each sample can be obtained.

In order to improve the overall accuracy of sequencing, particularly if quantification of a variant is required, UMIs can be used. These are short 'unique' nucleic acid sequences which are ligated to each individual DNA molecule prior to any amplification during library preparation such that every input DNA molecule has its own UMI sequence and can be separately identified. UMIs are particularly useful if very little input nucleic acid is available and the likelihood of large numbers of PCR repeats is high, or if very high-depth sequencing (e.g. tens or hundreds of thousands of reads) is occurring to look for rare variants e.g. in measurable residual disease (MRD) monitoring, where an early error may falsely bias the detection of MRD.

Short-read technologies

As discussed above, short-read technologies relate to those which sequence nucleic acid fragments typically up to 300 bp in length. A number of different proprietary technologies exist with those used by Illumina (see Figure 8.1a) and Thermo Fisher Scientific (see Figure 8.1b) currently most commonly deployed for clinical use. Both these technologies use a form of sequencing by synthesis, Illumina utilises the incorporation of fluorescently labelled complementary nucleotides, whereas Thermo Fisher Scientific exploits the release of hydrogen ions upon incorporation of (a) complementary nucleotide(s).

Figure 8.1a outlines Illumina's technology: The DNA molecules for sequencing, generated during library preparation, contain adaptors at both ends which allow them to bind to complementary primers adhered to channels on a flow cell. Each bound DNA molecule forms a template allowing synthesis of a new DNA molecule, contiguous with the adapter adhered to the flow cell. This newly synthesised DNA molecule can then bend so the end not adhered to the flow cell can interact with another primer, forming a bridge. Synthesis of a new DNA strand, again contiguous with the primer, results in a forward and reverse strand derived from each DNA molecule bound to the flow cell. These DNA molecules then undergo clonal amplification to form a cluster where there are ~1000 identical DNA molecules which originated from a single starting DNA molecule.

To determine the sequence of the bases in the DNA strands, a new strand is synthesised nucleotide by nucleotide by adding fluorescently labelled reversible terminator deoxynucleotide triphosphate molecules (RT-dNTPs), primers and polymerase. Owing to the nucleotides being terminator dNTPs only a single nucleotide can bind to a single fluorescence colour (different RT-dNTPs are labelled with a different colour) which is captured by a camera and correlates with a specific nucleotide. After each synthesis cycle, the fluorescent dye and blocking group (which is responsible for limiting nucleotide incorporation to one at a time) are enzymatically cleaved off allowing another RT-dNTP to be incorporated during the next cycle. This process is repeated until the desired length of the DNA molecule is sequenced with the order of bases being determined by the order of the different wavelengths of light emitted. Typically for clinical applications 'paired-end' sequencing is undertaken during which DNA molecules are sequenced in both forward and reverse directions; this allows improved accuracy of sequencing, particularly of repetitive regions and insertion/deletion variants. Illumina sequencers vary in the amount of sequencing data a single chip can produce from an iSeq to a NovaSeq with a NovaSeq able to analyse multiple whole genomes simultaneously.

Figure 8.1b outlines Thermo Fisher Scientific's technology: The DNA molecules for sequencing, generated during library preparation, include an adaptor that contains biotin. Biotin-containing DNA molecules are mixed with streptavidin-coated magnetic beads at a ratio such that ~one DNA molecule will bind to each bead (the streptavidin:biotin interaction is one of the strongest non-covalent interactions in nature). The DNA molecule is then clonally amplified via an emulsion PCR (emPCR) resulting in multiple copies of identical DNA molecules attached to each bead; emPCR allows each DNA molecule to be amplified separately in a water-in-oil droplet, preventing interaction between the different starting template DNA molecules and formation of chimeric reads. Beads covered with clonally amplified DNA molecules are then applied to a semiconductor chip covered with millions of microwells, at a concentration which means ~one DNA molecule is in each microwell.

To determine the sequence of the bases in the template DNA molecules, a new complementary DNA strand is synthesised. Different types of dNTP (i.e. dATP, dCTP, dGTP, dTTP) are sequentially flooded across the microwells in the chip. If a dNTP is complementary to the nucleotide on the DNA template molecule it will be incorporated into the new DNA strand which results in the release of a hydrogen ion and a decrease in the pH. The semiconductor chip is able to detect this pH change in the microwell and record which nucleotide it occurred in response to exposure. The release of hydrogen ions and change in pH is proportional to the number of consecutive identical nucleotides; i.e. two adjacent identical nucleotides will result in a doubling of the decrease in pH when their complementary nucleotide is incorporated into the growing DNA strand. Unattached dNTPs are washed out of the microwells and sequential exposure of the DNA template molecule to dNTPs continues until the desired length of the DNA molecule is sequenced. Thermo Fisher Scientific sequencers include the GeneStudio S5 and Genexus.

Long-read technologies

Long-read sequencing technologies usually refer to those that are able to sequence nucleic acid fragments 10 kb or longer. Using short-read technologies, it is difficult to accurately sequence highly repetitive regions of the genome as are seen in telomeres, centromeres and acrocentric genomic regions (i.e. where the centromere is located near the end of a chromosome rather than in the middle) which can make calling of structural variants troublesome owing to difficulties mapping (i.e. matching) short reads against a reference genome (see bioinformatics section for more information). Longer sequencing reads to reduce the possibilities of where a fragment of nucleic acid could match in the reference genome making mapping easier. Similarly with short-read sequencing if there is more than one variant in a gene, unless they are covered by the same read it is not possible to determine whether they are occurring in cis (i.e. are in the same allele) or in trans (i.e. are in different alleles); ability to accurately phase variants is relevant when the clinical implications of mono- or biallelic disruption of a gene differ (e.g. *TP53* mutations).

At the time of writing, long-read sequencing technologies are less mature in standard clinical practice but are under active investigation as to how and when they could be more routinely utilised. As with short-read technologies, a number of different long-read proprietary technologies exist with those used by Oxford Nanopore Technologies (ONT) (see Figure 8.1c) and Pacific Biosciences (PacBio) (see Figure 8.1d) being the most dominant.

Bioinformatic processing and interpretation of data

Bioinformatics pipeline

Once the sequencing itself has been performed, significant manipulation of the resulting raw output signal is required in order to have usable data that can influence patient care (see Figure 8.3). The initial stage is to convert the raw sequencing signal into corresponding nucleotide calls with each nucleotide usually accompanied by a quality score reflecting the likelihood it has been incorrectly called. This data is represented in the FASTQ file.

Following nucleotide calling, it is possible to decode the indexes applied to the reads and data can be demultiplexed and assigned to separate individuals. Adaptor and other 'added' sequences can also be computationally 'trimmed' i.e. removed at this point. Given in clinical practice the purpose of NGS is usually to identify 'differences from normal', data is normally aligned against a reference human sequence. It should be noted that a number of different human genome builds exist; as sequencing technologies improve and more human genomes are sequenced, sequentially 'more accurate' versions of the reference sequence have been produced. Care must be taken as to which build alignment is taking place against.

A number of different algorithms exist for performing this alignment each of which may perform better in different contexts (e.g. lower depth of coverage, frequent repetitive regions or sections with very high or low GC content). Depending on the nature of the sequencing and variant detection required, more than one alignment tool may be applied (see Figure 8.3). For example, owing to their repetitive similarity to the reference genome, FLT3 internal tandem duplications [ITDs] are often difficult to align using standard alignment tools with short-read sequencing; Specialist programmes such as PINDEL are able to take unaligned reads and perform a local re-alignment in order to detect this variant type. The output of the alignment process is a BAM file (or closely related SAM or CRAM file).

After sequencing data has been aligned to a reference genome, variant calling can be undertaken which seeks to identify 'differences from normal'. As with aligners, a number of

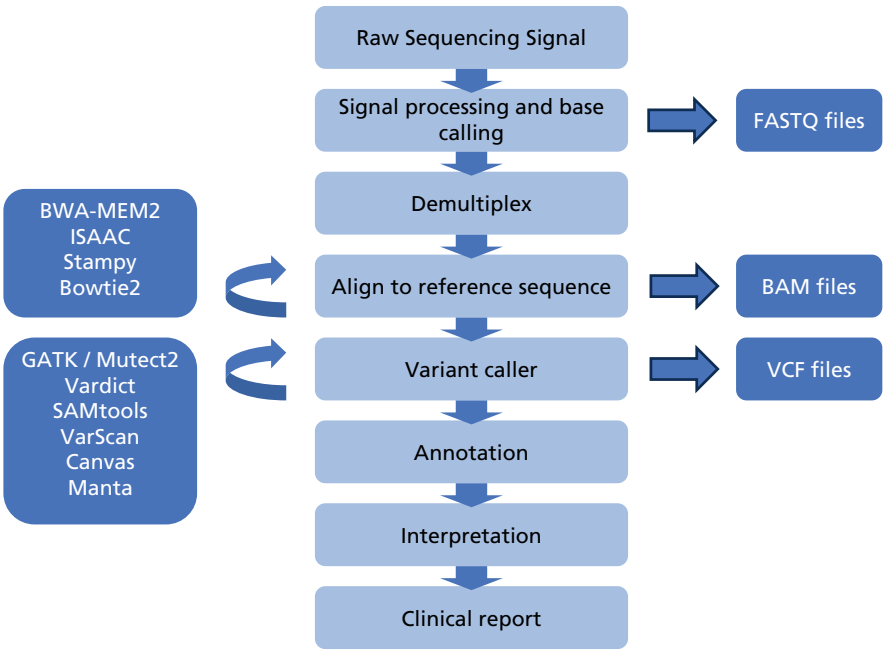

Figure 8.3 Flow chart demonstrating the different processing steps raw sequencing data needs to undergo before reaching a clinically useful output. Indicative examples of alignment tools and variant callers have been given but it should be noted that many alternative tools, not listed, are also available.

different variant calling algorithms are available which may perform better in specific sequence contexts (as discussed above) and with the detection of particular variant types (e.g. SNVs, CNVs, SVs). In clinical practice, it is advised to use a combination of variant callers in order to increase the sensitivity of variant detection. The output of the variant calling process is the VCF file.

Having identified variants, it is necessary to annotate them in order to understand what contribution, if any, they may be having to a presenting phenotype. A number of different annotation tools exist (e.g. ANNOVAR, Variant Effect Predictor [VEP]) which seek to describe the effect a variant is having. This includes where the variant occurs relative to a gene (e.g. exonic, intronic, splice site) and, assuming a variant is in a coding region, these include information about the transcript involved (multiple mRNA transcripts can be produced from a single gene). Annotation describes the effect the variant is expected to have (e.g. amino acid substitution, introduction of a premature termination codon, in-frame deletion, balanced translocation) and can include information from computational algorithms as to the likely predicted effect of the variant i.e. deleterious/tolerated (e.g. SIFT, PolyPhen2).

It also provides information about the number of reads supporting the presence of a variant from which the variant allele frequency can be derived. This information can attempt to assign germline or somatic status although it should be noted that if there is a high tumour content in a sample, acquired variants can have an allele frequency similar to that of a germline variant. Variant annotation can be more extensive and provide information on its frequency in germline population databases (e.g. gnomAD/1000 Genomes), as well as databases listing variants associated with specific diseases (e.g. Human Gene Mutation Database [HGMD] for inherited i.e. germline diseases and Catalogue of Somatic Mutations in Cancer [COSMIC] for malignant diseases).

Interpretation of variants

Once a variant has been confidently identified, investigation is required to assign pathogenicity or otherwise. Increasingly this is being automated at the end of the bioinformatics pipeline by the utilization of highly curated databases (e.g. HGMD in inherited disease and OncoKB in malignant disease), but in the creation of these databases, extensive manual assessment has typically taken place. In order to ensure consistent interpretation of variants between different operators, a number of variant classification systems have emerged. The first was developed by the American College of Medical Genetics (ACMG) and Association for Molecular Pathology (AMP) and considered variants in the context of their likely causation of Mendelian disorders. Variants were therefore classified as pathogenic, likely pathogenic, uncertain significance, likely benign and benign based on the available evidence (e.g. population frequency, functional studies, segregation data) as to their contribution to a specific phenotype.

Although this initial classification system is well suited to inherited disease, it works less well with malignant conditions as in this context, frequently the detection of genomic abnormalities is not utilised to make the diagnosis (which can be done morphologically or immunophenotypically), but rather to inform prognosis and treatment (see utility section below). Therefore, a classification system based on actionability was developed by the AMP, American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) which divided variants into four tiers (i): Strong clinical significance, (ii): Potential clinical significance, (iii): Unknown clinical significance, IV: Benign or likely benign.

While the AMP/ASCO/CAP framework provided a mechanism for accurately assigning clinical actionability to a somatic variant, it did not provide a fully comprehensive system for determining the oncogenicity of an acquired variant. Therefore, the Clinical Genome Resource (ClinGen), Cancer Genomics Consortium (CGC) and Variant Interpretation for Cancer Consortium (VICC) adapted the ACMG/AMP guidelines specifically for somatic variants and developed a similar five-category classification system: oncogenic, likely oncogenic, uncertain significance, likely benign and benign. Using a combination of population, functional, predictive, cancer hotspot and computational data, a numerical score is generated which assigns a variant to one of the five categories. This variant classification system is increasingly being adopted across laboratories at the time of writing with the hoped effect of more consistent variant interpretation throughout the world.

Utility

The potential utility of NGS can be categorised in a similar way to that of any other genomic testing methodology i.e. performed for diagnostic, prognostic, therapeutic (including the selection of targeted inhibitors), MRD and genetic predisposition identification purposes. Although individual chapters elsewhere in this book cover the genomics of specific haematological diseases, below is a summary of examples in each of these utility categories.

Diagnostic

In the context of inherited non-malignant haematological disorders, the availability of NGS has enhanced the ability to reach a diagnosis in a timely fashion owing to being able to sequence multiple genes pertaining to the spectrum of differential diagnoses raised by the presenting phenotype. Whereas previously sequential rounds of Sanger sequencing +/− Multiplex Ligation-dependent Probe Amplification (MLPA) could be required to reach (or eliminate) the suspected diagnoses, a single NGS panel or WES with virtual panel application can provide similar information in a single assay. Table 8.2 lists the different panels (of 3 or more genes) currently (in 2023) commissioned for inherited haematological disorders, by the Genomic Medicine Service (GMS) in England's National Health Service (NHS).

Accepting that acquired haematological disorders (usually malignancies) are often diagnosed through a combination of clinical presentation, morphology and immunophenotyping, genomic information can still be critical for making diagnoses in both acute (e.g. acute promyelocytic leukaemia [APL]) and chronic presentations (e.g. myeloproliferative neoplasms). Although to date, the urgency of diagnosis in acute leukaemias and usual turnaround times (TATs) of NGS have meant Fluorescence *In Situ* Hybridization (FISH) testing is a more practical approach (e.g. to detect *PML::RARA*). In less urgent presentations (e.g. most suspected myeloproliferative neoplasms) NGS has utility in reaching or eliminating a diagnosis in an efficient manner without the need for sequential rounds of testing.

The recently published 5th edition of the World Health Organization (WHO) Classification of Haematolymphoid Tumours and International Consensus Classification (ICC) of Myeloid and Lymphoid Neoplasms (Chapters 20 & 23–26) both demonstrate the need for comprehensive genomic information to reach accurate diagnoses in haematological malignancies. It should also be noted that NGS is key to the diagnosis of acquired pre-malignant disorders such as Clonal Haematopoiesis of Indeterminate Potential (CHIP) and Clonal Cytopenia(s) of Undetermined Significance (CCUS) (Chapter 38) as well as acquired non-malignant disorders which can present with haematological phenotypes such as VEXAS syndrome (associated with mutations in the *UBA1* gene and presenting with similarities to myelodysplasia). Table 8.3 part A provides examples of the role of NGS in the diagnosis of haematological malignancies.

Prognostic

Although NGS is primarily undertaken in suspected inherited non-malignant haematological disorders for diagnostic purposes, associations are increasingly being demonstrated between genotype and prognosis. For example, the specific mutation in the *F8* gene contributes to an individual's risk of acquiring FVIII inhibitors in haemophilia A, whereas the genotype in Fanconi anaemia has implications for both phenotype and the risk of bone marrow failure. In contrast, in haematological malignancies, much of the NGS undertaken is primarily for prognostic purposes and genomically-defined diagnoses in the WHO and ICC have often been selected because of a specific associated prognosis; e.g. acute myeloid leukaemia (AML) with *NPM1* mutation and AML with myelodysplasia-related gene mutations are associated with favourable and adverse prognoses respectively. Furthermore, refinement of morphological diseases into multiple genomic subtypes with particularly associated prognoses has only become possible with the widespread implementation of NGS and subsequent correlation of genotype with clinical outcomes.

Table 8.2 List of gene panels (with 3 or more target genes) currently commissioned with the NHS in England for inherited haematological disorders. The panels, although correct at the time of writing, are regularly updated with the most up-to-date panel content available at https://nhsgms-panelapp.genomicsengland.co.uk/panels.

Panel	Gene content
Bleeding and platelet disorders	*ABCG5, ABCG8, ACTB, ACTN1, ACVRL1, ANKRD26, ANO6, AP3B1, AP3D1, ARPC1B, BLOC1S3, BLOC1S6, CDC42, CHST14, COL3A1, CYCS, DIAPH1, DTNBP1, ENG, ETV6, F10, F11, F12, F13A1, F13B, F2, F5, F7, F8, F9, FERMT3, FGA, FGB, FGG, FLI1, FLNA, FYB1, GATA1, GBA, GFI1B, GGCX, GNE, GP1BA, GP1BB, GP6, GP9, HPS1, HPS3, HPS4, HPS5, HPS6, IKZF5, ITGA2B, ITGB3, KDSR, KLKB1, KNG1, LMAN1, LYST, MCFD2, MECOM, MPIG6B, MPL, MYH9, NBEAL2, P2RY12, PLA2G4A, PLAU, PTGS1, RASGRP2, RBM8A, RUNX1, SERPINE1, SERPINF2, SLFN14, SRC, STIM1, STXBP2, TBXA2R, TBXAS1, THBD, THPO, TNXB, TPM4, TUBB1, VIPAS39, VKORC1, VPS33B, VWF, WAS*
Fanconi anaemia or bloom syndrome	*BLM, BRCA1, BRCA2, BRIP1, ERCC4, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, PALB2, RAD51, SLX4, TOP3A, UBE2T*
Cytopenia – not Fanconi anaemia	*ACD, ADA2, AK2, ANKRD26, CLPB, CSF3R, CTC1, CXCR4, CYCS, DDX41, DKC1, DNAJC21, EFL1, ELANE, ERCC6L2, ETV6, FYB1, G6PC3, GATA1, GATA2, GFI1, HAX1, JAGN1, KLF1, MECOM, MPL, MYSM1, NHP2, PARN, RMRP, RPA1, RPL11, RPL15, RPL35A, RPL5, RPS10, RPS17, RPS19, RPS24, RPS26, RPS29, RPS7, RTEL1, SAMD9, SAMD9L, SBDS, SRC, SRP54, STN, TAZ, TERC, TERT, THPO, TINF2, USB1, VPS45, WAS, WIPF1, WRAP53*
Hereditary Erythrocytosis	*BPGM, EGLN1, EPAS1, EPO, EPOR, HBA1, HBA2, HBB, SLC30A10, VHL*
Inherited predisposition to acute myeloid leukaemia	*ANKRD26, CEBPA, DDX41, ETV6, GATA2, MBD4, RUNX1, SAMD9L, TERC, TERT, TP53*
Iron metabolism disorders – NOT common HFE mutations	*ABCB7, ALAS2, ATP7B, BMP6, CP, CYBRD1, FTL, GBA, GLRX5, HAMP, HFE, HFE2, SLC11A2, SLC25A38, SLC40A1, TF, TFR2, TMPRSS6*
Rare anaemia	*ABCB7, ABCG5, ABCG8, ADA2, AK1, ALAS2, ALDOA, AMN, ANK1, C15orf41, CD59, CDAN1, CUBN, CYB5R3, DHFR, EPB41, EPB42, G6PD, GATA1, GCLC, GIF, GLRX5, GPI, GSR, GSS, HBA1, HBA2, HBB, HBD, HBG1, HBG2, HEATR3, HK1, HSPA9, KCNN4, KIF23, KLF1, LARS2, LPIN2, MTR, MTRR, NT5C3A, PFKM, PIEZO1, PKLR, PUS1, RHAG, RPL11, RPL15, RPL31, RPL35A, RPL5, RPL9, RPS10, RPS17, RPS19, RPS24, RPS26, RPS29, RPS7, SBDS, SEC23B, SLC11A2, SLC19A2, SLC25A38, SLC2A1, SLC4A1, SPTA1, SPTB. TCN2, TF, TMPRSS6, TPI1, TRNT1, UMPS, VPS4A, XK, YARS2*
Thrombocythaemia	*JAK2, MPL, THPO*
Thrombophilia with a likely monogenic cause	*ADAMTS13, F2, F5, FGA, FGB, FGG, HRG, PIGA, PLG, PROC, PROS1, SERPINC1, SERPIND1, SERPINE1, THBD*

The broad deployment of NGS in clinical trials has allowed the addition of molecular (i.e. gene level) genomic data to prognostic scoring systems. Such comprehensive risk calculators include the molecular international prognostic scoring system (IPSS-m) and the mutation-enhanced international prognostic scoring system for transplant-age patients (MIPSS70) in myelodysplasia and primary myelofibrosis respectively. Internationally, there is ongoing work in the development of similar genomically-driven prognostic scoring systems in lymphomas (e.g. LymphGen in diffuse large B cell lymphoma which uses CNV and SNV data): However, the requirement to date for high-quality DNA derived from fresh rather than FFPE tissue for these NGS assays has limited the widespread use of NGS in this context. Table 8.3 part B provides examples of the role of NGS in the prognostication of haematological malignancies.

Therapeutic

NGS data can be considered to inform treatment strategies in a range of scenarios in haematological malignancies. If NGS has been used to make or subtype a diagnosis e.g. assist in the differentiation of a morphologically unusual hairy cell leukaemia from lymphoplasmacytic lymphoma or another mature B cell neoplasm (via the detection or not of *BRAF* p.[Val600Glu] or *MYD88* [Leu265Pro] variants), each of which will have its own treatment

Table 8.3 Gives illustrative examples of where NGS is used in clinical practice. It is acknowledged that not all laboratories will be delivering testing for all of these targets using NGS approaches. It is recognised that many targets can be delivered using different NGS assays e.g. DNA or RNA panels or WGS and that the preferred approach as well as the spectrum of targets is likely to evolve over time. It should be noted that the RNA panels referred in Figure 8.3 all used cDNA rather than native RNA as their substrate.

(A) Examples of how NGS is used to assist in the diagnosis of haematological malignancies. In general the target lists are not exhausted and should be supplemented by reference to the most recent malignancy-specific diagnostic guidance.

PART A: DIAGNOSTIC TESTING

Clinical indication	NGS assay	Targets
Acute myeloid leukaemia	RNA panel	*BCR::ABL1* *CBFB::MYH11, RUNX1::RUNX1T1* *DEK::NUP214* *GATA2::MECOM(EVI1)* and other *MECOM* rearrangements *MLLT3::KMT2A* and other *KMT2A* rearrangements *NUP98* rearrangements *PML::RARA* and other *RARA* rearrangements *RBM15::MRTFA*
	DNA panel	*ASXL1, BCOR, CEBPA, EZH2, NPM1, RUNX1, SF3B1, SRSF2, STAG2, TP53, U2AF1, ZRSR2* del(5q)/add(5q), −7/del(7q), +8, del(12p)/add(12p), −17/add(17p) or del(17p), del(20q)
	WGS	All of the above and complex karyotype, t(5q), t(12p), i(17q), idic(X)(q13)
Acute lymphoblastic leukaemia	RNA panel	*BCR::ABL1* *KMT2A* rearrangements *ETV6::RUNX1* *IL3::IGH* *TCF3::PBX1* ABL-class rearranged *MYC* rearrangement *MEF2D* rearrangement *ZNF384* rearrangement *NUTM1* rearrangement *HLF* rearrangement *UBTF::ATXN7L3/PAN3,CDX2* *BCL11B* rearrangement
	DNA panel	*IKZF1, PAX5* iAMP21, hyperdiploid, hypodiploid, near haploid
	WGS	All of the above and *DUX4* rearrangement
Suspected myeloproliferative neoplasm (not chronic myeloid leukaemia)	DNA panel	*CALR, JAK2, MPL* *CSF3R* *ASXL1, EZH2, IDH1, IDH2, SF3B1, SRSF2, TET2* and others*
Mature B cell neoplasms (e.g. hairy cell leukaemia, hairy cell leukaemia variant, lymphoplasmacytic lymphoma)	DNA panel	*BRAF, MAP2K1, MYD88*

* Other myeloid-malignancy related genes can be added to the list of genes where the presence of mutations can be used to indicate clonal haematopoiesis in the context of a suspected diagnosis of a myeloproliferative neoplasm.

(*Continued*)

Table 8.3 (*Continued*)

(B) Examples of how NGS is used to inform prognosis in haematological malignancies. It should be noted that many other genomically-informed prognostication algorithms exist beyond those listed in the following table and it is expected that the contents of these will evolve over time and additional data becomes available and treatments change.		
PART B: PROGNOSTIC TESTING		
Clinical indication	**NGS assay**	**Targets**
Acute myeloid leukaemia	RNA panel	*BCR::ABL1* *CBFB::MYH11, RUNX1::RUNX1T1* *DEK::NUP214* *GATA2::MECOM(EVI1)* and other *MECOM* rearrangements *KAT6A::CREBBP* *MLLT3::KMT2A* and other *KMT2A* rearrangements
	DNA panel	*ASXL1, BCOR, CEBPA, EZH2, FLT3, NPM1, RUNX1, SF3B1, SRSF2, STAG2, TP53, U2AF1, ZRSR2* −5/del(5q), −7, −17/abn(17p)
	WGS	All of the above and complex karyotype
Myelodysplasia	DNA panel	*ASXL1, BCOR, BCORL1, CBL, CEBPA, DNMT3A, ETNK1, ETV6, EZH2, FLT3, GATA2, GNB1, IDH1, IDH2, KRAS, KMT2A* (PTD), *NPM1, NF1, NRAS, PHF6, PPM1D, PRPF8, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAG2, TP53, U2AF1, WT1* del(5q), −7/del(7q), +8, del(11q), del(12p), +19, del(20q), −Y
	RNA panel	*GATA2::MECOM(EVI1)* and other *MECOM* rearrangements
	WGS	All of the above and complex karyotype, i(17q),
Primary myelofibrosis	DNA panel	*ASXL1, CALR, CBL, EZH2, IDH1, IDH2, JAK2, KRAS, NRAS, SRSF2, TP53, U2AF1*
	WGS	All of the above and prognostic cytogenetic abnormalities
(C) Examples of how NGS is used to inform therapy haematological malignancies. This table has been limited to targeted inhibitors but it should be noted that genomic data is also used to decide on the choice of non-targeted treatments, as well as the intensity of treatment and whether an allogeneic HSCT is required.		
PART C: THERAPEUTIC TESTING		
Clinical indication	**NGS assay**	**Targets**
Acute myeloid leukaemia	RNA panel	*KMT2A* rearrangements – patient may respond to menin inhibitors
	DNA panel	*FLT3* – patients with an activating mutation may respond to a FLT3-inhibitor *NPM1* – patients with a 4 bp insertion in exon 11 may respond to a menin inhibitor *IDH1* – patients with a codon 132 mutation may respond to an IDH1-inhibitor *IDH2* – patients with a codon 140 or 172 mutation may respond to an IDH2-inhibitor
	WGS	All of the above
Chronic myeloid leukaemia	DNA panel	*BCR::ABL1* TKD – identity of any mutations in the TKD or the *BCR::ABL1* fusion genes informs tyrosine kinase inhibitor choice

Table 8.3 (*Continued*)

Myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions	RNA panel	*FIP1L1::PDGFRA* and other *PDGFRA* rearrangements – patient may respond to tyrosine kinase inhibitor *ETV6::PDGFRB* and other *PDGFRB* rearrangements – patient may respond to tyrosine kinase inhibitor *ZMYM2::FGFR1* and other *FGFR1* rearrangements – patient may respond to FGFR inhibitor *PCM1::JAK2* and other *JAK2* rearrangements – patient may respond to JAK inhibitor *ETV6::FLT3* and other FLT3 rearrangements – patient may respond to FLT3 inhibitor *ETV6::ABL1* – patient may respond to second generation tyrosine kinase inhibitor
	WGS	All of the above
Follicular lymphoma	DNA panel	*EZH2* – patients with an activating mutation may respond to an EZH2-inhibitor
(D) Examples of how NGS can be used in both MRD target identification and MRD quantification assays.		
PART D: MEASURABLE RESIDUAL DISEASE TESTING		
Target identification		
Clinical indication	**NGS assay**	**Targets**
Acute myeloid leukaemia	RNA panel	*PML::RARA* *CBFB::MYH11, RUNX1::RUNX1T1* *KMT2A* rearrangements
	DNA panel	*KMT2A* rearrangements *NPM1* 4 bp insertion in exon 11
	WGS	All of the above
Acute lymphoblastic leukaemia	DNA panel	*IGH, IGK, TCRB, TCRD, TCRG*
	WGS	All of the above
Measurable residual disease detection		
Lymphoid malignancies	DNA panel	*IGH, IGK, TCRB, TCRD, TCRG*
(E) Examples of how NGS can be used for the detection of germline predisposition to haematological malignancies.		
PART E: GERMLINE PREDISPOSITION TESTING		
Clinical indication	**NGS assay**	**Targets**
Myeloid malignancies	DNA panel	*ANKRD26, CBL, CEBPA, DDX41, ETV6, GATA2, KRAS, NRAS, PTPN11, RUNX1, SAMD9, SAMD9L, TP53* Genes associated with bone marrow failure syndromes see table 2
	WGS	All of the above
Lymphoid malignancies	DNA panel	*DDX41, ETV6, IKZF1, PAX5, RUNX1, TP53*
	WGS	All of the above

approaches, it has indirectly directed therapy. Similarly, if NGS has been used to refine a diagnosis of acute myeloid leukaemia and demonstrate a likely genomic background of myelodysplasia this will influence the choice of front-line therapy (e.g. eligible for CPX-351). Furthermore, the use of genomic data by the 2022 ELN risk classification by genetics at initial diagnosis is routinely used in AML to help inform the decision around an allogeneic haematopoietic stem cell transplant (HSCT) in otherwise suitable patients in complete remission 1 (CR1).

The demonstration of a specific genomic abnormality using NGS may lead to the use of a targeted inhibitor e.g. *FLT3*, *EZH2* and menin inhibitors in the context of activating *FLT3*

mutations, activating *EZH2* mutations and *NPM1* exon 11 4 bp insertions or *KMT2A* disruptions (rearrangements or partial tandem duplications [PTD]) respectively. NGS assays can also be used to investigate for secondary resistance mutations as seen in the tyrosine kinase (TK) domain of the *BCR::ABL1* fusion gene in the context of TK inhibitor usage in CML. Table 8.3 part C provides examples of the role of NGS in therapy decisions in haematological malignancies.

Measurable residual disease

When considering MRD, NGS is currently primarily used for target identification. In myeloid malignancies, much current MRD monitoring involves the detection of translocations e.g. *BCR::ABL1* in CML and *RUNX1::RUNX1T1* in AML using reverse transcriptase quantitative PCR (RT-qPCR) or digital droplet PCR (ddPCR) assays. In order to use such a test, it is necessary to know where the respective breakpoints are, something which can be determined by both DNA and cDNA assays as described above. Other MRD targets include the 4 bp insertion in exon 11 of the *NPM1* gene, the identity of which (as there are multiple different reported 4 bp insertions) is usually determined using NGS. In lymphoid malignancies, the basis of MRD assays is usually immunoglobulin (IG) (*IGH*, *IGK*) or T cell receptor (TCR) (*TCRB*, *TCRD*, *TCRG*) VDJ or VJ rearrangements. NGS is increasingly being used as the preferred method for this target identification as opposed to Sanger sequencing-based approaches.

In addition to using NGS for the identification of MRD targets, the technology can also be used for the detection of MRD itself providing the sequencing is of high enough depth (i.e. up to hundreds of thousands of reads) and methodology is in place to remove PCR repeats e.g. the use of UMIs. Commercial NGS assays exist for the detection of IG and TCR rearrangement MRD with sensitivities quoted of up to 1×10^{-6} which is 1–2 logs more sensitive than non-NGS MRD assays. More recently, in AML, there has been interest in using UMI-assisted NGS-based MRD assays to look at the clinical significance of the detection of a range of other SNVs. Currently, this remains largely a research tool as it is unclear which SNVs in which genes represent MRD associated with a poor prognosis. MRD is usually predicated on detecting the initial driver mutation(s) which are stable throughout the lifecycle of the malignant clone (as opposed to latterly acquired sub-clonal mutation[s]), yet there is some evidence that persistence of variants associated with CHIP does not confer an inferior prognosis. Table 8.3 part D provides examples of the role of NGS in MRD monitoring in haematological malignancies.

Germline predisposition

Wider use of NGS in haematological malignancies in both the research and clinical settings has led to the discovery of an increasing number of genomic abnormalities which are associated with a predisposition to haematological malignancies, currently best characterised for acute leukaemias. It follows, therefore, that NGS is an efficient way of detecting germline variants conferring a predisposition to haematological malignancies. The caveat to this is that most clinical NGS is performed using tumour samples without a comparative germline sample meaning it is not possible to definitively determine the origin of a variant (something that would require the testing of separate germline sample). This topic will be further discussed below. Table 8.3 part E provides examples of the role of NGS in the detection of germline predisposition to haematological malignancies.

Discovery

The categories of utility listed above all relate to the deployment of NGS in the routine clinical setting. However, as discussed, much of our knowledge which has precipitated this utility has arisen from the use of NGS in the research setting. Therefore, the use of NGS, particularly those assays with a broader footprint such as WGS, WES and whole RNA-seq, retains an important discovery purpose to enhance our understanding of the genomics of haematological diseases, both malignant and non-malignant. Efforts should, therefore, be made to try and enable the required ethical governance and logistical frameworks to be in place to facilitate appropriate anonymised sharing of NGS results and associated clinical phenotype to create datasets which can inform our knowledge and treatment of these diseases.

Practical considerations

As with any diagnostic technology, its utilization in the clinical setting depends on its ability to deliver the required information in the required timescale at the right price. The following section discusses these and other considerations which help inform how and when this technology is deployed.

Cost

The cost of any individual NGS assay will depend on the size of its footprint, the depth of sequencing required, the specific NGS technology being used and the number of samples being multiplexed. Typically, the larger the footprint, deeper the sequencing and lower the number of samples that can be multiplexed, the higher the cost of the assay on a per-patient basis. As discussed above, the footprint and depth of an assay will be related to the questions the assay is trying to answer and the expected allele frequency of an abnormality. In addition to the size of assay footprint and required depth, the number of samples that can be multiplexed is influenced by the sequencer choice, size of the

sequencing flow cell or chip being used and available sample numbers. Generally, the larger the flow cell the cheaper the per base cost of sequencing, but filling the largest available requires availability of sufficient numbers of samples from a single or compatible assays. In high throughput laboratories, this does not usually present a problem, but in smaller laboratories, the requirement for batching to ensure the economic viability of an assay can compromise the turn-around time such that a result is not available in a clinically meaningful timescale.

Other costs to consider are the technician's hands-on time during library preparation and scientist analysis and interpretation of the resulting sequencing data. Automation of both assay setup and data interpretation can result in cost savings. A number of commercial interpretation and decision support programmes exist for some types of genomic data to try and streamline the reporting process. It should be noted that the general direction of costs for NGS assays on a per-base basis is downwards.

Turn-around-times

The TAT of any NGS depends on the technology, degree of automation and complexity of the library preparation, bioinformatics pipeline and analysis process. ONT technologies have examples of assays returning results in minutes to hours, while most NGS assay workflows can return results in hours to 3 days (including WGS). It is notable that most clinical services have longer TATs which reflects the need for economic batching of samples, availability of technicians in non-automated workflows, access to sequencers and the time often taken to perform analysis of the data and clinical interpretation. Greater degrees of consolidation of services, automation of laboratory workflows and the greater use of machine learning in interpretation are likely to reduce the TATs.

Interpretation complexities

Prior to the advent and introduction of NGS, most clinical molecular genomic testing was limited to investigating the presence or absence of well-known genomic abnormalities which resulted in reporting being a binary exercise i.e. abnormality detected or not detected. The increasing breadth of footprints of NGS assays means large amounts of data can be generated, for much of which the clinical significance is unclear. One approach is to apply restrictive bioinformatics filters to the data to limit at least the initial analysis to only the most well-characterised regions, particularly if a targeted panel assay is designed to answer different questions in different haematological phenotypes. As discussed above variant interpretation frameworks in both the germline and acquired settings exist to assist with this interpretation but both inevitably result in the classification of many variants as of uncertain significance (VUS). There still remains some inter and even intra-laboratory variability as to which VUSs, if any, should be reported back to a clinician. In the context of a suspected haematological malignancy, the reporting of a VUS with an allele frequency suggestive of an acquired variant (e.g. <30%) can be useful as an indication of clonality. The observation that NGS assays relatively frequently demonstrate such clonality in the blood particularly of ageing individuals, has helped contribute to the coining of the pre-malignant diagnoses of CHIP and CCUS. Detecting such an abnormality without knowing the full implications can cause anxiety for patient and healthcare professionals alike (see clonal haematopoiesis Chapter 38 for more details).

Other examples of where the availability of NGS data can create challenges for the patient and healthcare team are in the MRD setting (as discussed above) and the germline predisposition setting. Comprehensive data collection on the long-term outcomes of individuals with variants in different genes persisting post-treatment (e.g. for AML) is needed in order to know when this should precipitate an intervention; e.g. in the setting of residual low-risk CHIP following AML therapy. How to manage the detection of likely germline variants in certain haematological predisposition genes e.g. *DDX41* has also created a management dilemma. Although offering, and if accepted, consenting the patient for confirmatory testing using an appropriate germline sample is straightforward, how and when to cascade testing in the wider family and how to manage the detection of a pathogenic variant in any family members in terms of ongoing surveillance remains an evolving field that will require additional data.

Finally, how to retrofit high-resolution NGS data (particularly that including structural abnormalities) to prognostic classifications developed using less sensitive technologies such as karyotyping and FISH is currently unclear. Definitions such as a complex karyotype in AML, critical for prognostication, relate to the low-resolution technique of karyotyping (resolution 5–10 Mb). That equates to a complex karyotype with NGS techniques with individual base-level resolution remains a research question and is only likely to be answered by considerable genotype:phenotype and clinical outcome correlation.

Ethical considerations

Although healthcare systems have developed systems of up-front informed consent for all research genomic testing and designated germline testing in the clinical setting, there is much less uniformity around clinical practice in the acquired setting. When genomic testing was confined to a limited set of well-characterised mutations, the probability of detecting an abnormality with unexpected implications was low. As the footprint of even targeted assays has grown, the probability of detecting a

variant which could be germline in origin has increased with the subsequent uncertainties of how this should be optimally managed in the wider family setting as discussed above. Whether patients should be consented up-front for this possibility remains a controversial subject.

As laboratories move towards larger and larger NGS panels (and even routine WGS) which are designed to be used for both haematological and non-haematological malignancies, increasingly the footprint contains genes in which germline variants confer a predisposition to non-haematological malignancies. How to deal with the presence of such variants remains controversial; one approach is to apply virtual filters such that only those predisposition genes relevant to haematological malignancies are analysed, another approach is to obtain up-front patient consent for the presence of additional findings in such off-target genes. As NGS testing progresses over the next decade it is envisioned that a more consistent framework around how to deal with these challenges will evolve.

The future

The introduction of NGS into clinical haematological practice has been a game-changer. The ability to routinely undertake extensive genomic analysis has developed our ability to diagnose haematological diseases as well as highlighted a spectrum of pre-malignant disorders hitherto undiscovered. It has enhanced our ability to prognosticate, demonstrated targets for therapeutic intervention, improved monitoring of response to therapy and paved the way for potential earlier diagnosis through the detection of individuals with a genetic predisposition. However, as with any new technology, there are challenges and for NGS these include how to interpret the breadth of data and manage the associated uncertainty, and how to deploy clinically in an economical and timely fashion.

If the current trajectories continue, it would be envisioned that the cost of NGS will decrease and the time required for any assay will shorten. It would also be expected that with appropriate correlation of genomic data with clinical phenotype, our understanding of what the genomic data means on an individual patient level will improve, potentially assisted by artificial intelligence and machine learning.

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CHAPTER 9

The molecular basis of haematological malignancies

9

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Introduction

Cellular growth and differentiation are carefully controlled processes that are regulated by several interconnected pathways in order to facilitate diverse biological phenomena ranging from development, responses to normal and abnormal stimuli and the replacement of dying cells. In this context, the growth of individual cells is restrained in the interest of their cognate tissue and by extension of the organism as a whole. Oncogenesis represents the progressive corruption of this order and the stepwise escape of an individual cell and its progeny from checks on their growth. This corruption occurs in the form of the serial acquisition of genetic mutations, which disrupt the genome of the fateful cell and morph it into a *cancer genome* (Table 9.1). In turn, the ontogeny of the cell of origin and the nature of the mutations are the primary determinants of the cancer phenotype, including its histological type, biological behaviour, clinical characteristics and responsiveness to therapies.

Over the last few years, we have witnessed unprecedented progress in our understanding of cancer genomes, driven primarily by extraordinary advances in DNA sequencing technologies. The impact of cancer genomics on the study of haematological malignancies has been particularly dramatic, in large part because of our deep prior acquaintance with normal blood cell development and hierarchy, as well as our detailed characterisation and classification of haematological cancers. Importantly, these developments are not only enhancing our understanding of blood cancer biology but are already having an impact on the way we diagnose and treat patients.

This chapter will describe important advances in genomics and review key themes and paradigms relating to the pathogenesis and evolution of haematological cancers.

The cancer genome

The identification of the Philadelphia chromosome in patients with chronic myeloid leukaemia (CML) by Nowell and Hungerford in 1960 was the first report of a genetic lesion associated with a human malignancy and forms a landmark in cancer studies. It has since become clear that cancer is a genetic disease, which develops through a process of Darwinian-like clonal evolution involving the stepwise acquisition of somatic mutations in individual cells subject to natural selection on the basis of their ability to outgrow their peers through increased proliferation and survival. Within a healthy adult human, there are many small clones, most of which have limited abnormal growth potential and are invisible or manifest as common benign growths such as skin moles. Occasionally, however, a single cell acquires a set of mutations that allows it to proliferate to the extent that it generates a malignant phenotype. The nature of such mutations and the way in which they collaborate to produce the malignant phenotype can only be fully understood through the integrated study of the large set of variants within a tumour.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 9.1 Glossary.

Acquired mutation: see 'somatic mutation'

Allele burden: The fraction of alleles with a specific sequence in relation to the total number of alleles for the same region of the genome. For example, a heterozygous mutation in a pure population of leukaemia cells has an allele burden of 0.5. If 70% of cells are leukaemic and 30% of cells are normal, the mutant allele burden of the heterozygous mutation would be $0.7 \times 0.5 = 0.35$.

Amplification: A genetic modification producing an increased number of copies of a genomic region

Autosomal dominant: Inherited mutation affecting one allele on a non-sex chromosome is sufficient to confer the disease.

Autosomal recessive: Inherited mutation must affect both alleles on the respective non-sex chromosomes to confer the disease.

Branching evolution (of cancer): A form of clonal evolution of cancer which leads to the generation of more than one clone of cells characterised by distinct somatic mutations, but which share at least one mutation traceable back to a single ancestral cell.

Cancer genome: The genome of a cancer cell, which differs from the germline genome as a result of somatic mutations.

Chromosomal translocation: see 'genomic rearrangements'.

Chromothripsis: a single catastrophic event by which hundreds to thousands of chromosomal rearrangements occur in confined genomic regions of one or a few chromosomes (from Greek θρύΨις = shattering into small pieces).

Clonal evolution (of cancer): The stepwise acquisition of mutations in a founder cell and its progeny leading towards the development of a cancer.

Clonal mutation: A mutation present in a population of related cells derived from a single cell.

Constraint hypothesis: A hypothesis about clonal evolution proposing that the observed order of acquisition of somatic mutations during cancer evolution reflects a requirement for a specific mutation to occur before another for a growth advantage to be gained by the host cell(s).

Co-occurrence (of cancer mutations): The occurrence of two or more mutations in the same type of cancer more often than would be expected by chance.

Convergent evolution (of cancer): A pattern of cancer evolution during which independent clones expand after acquiring the same or very similar mutation. This is likely to reflect the fact that such a mutation is particularly advantageous to the specific cancer cell, giving a marked growth advantage when acquired by chance.

Deletion: A genetic modification leading to the loss of a genomic region.

Dominant negative mutation: A heterozygous mutation that leads to marked or complete loss of function of the coded protein and of the normal protein coded by the other (wild-type) copy of the gene.

Driver gene or driver mutation: A mutated gene that confers a selective growth advantage to a cancer cell

Epigenetics: The study of changes to DNA and chromatin, other than those that alter the DNA nucleotide sequence, that alter the transcriptional potential of a cell and are usually heritable

Exome: The collection of all exons in a genome.

Exome sequencing: Sequencing of all exons in a genome. This has referred to exons of protein-coding genes, but increasingly non-protein-coding genes are included (e.g. long non-coding RNAs).

Gain-of-function mutation: A mutation that gives the coded protein a novel or markedly enhanced function

Genome-wide association studies (GWAS): studies of many common and uncommon genetic variants in different individuals to determine if any variant is associated with a disease or trait. The primary outcome of these studies is the identification of variants such as SNPs which are *associated* with, but do not necessarily cause the disease in question.

Genomic instability: Increased frequency of mutations in the genome caused by either increased DNA damage or deficient DNA repair.

Genomic rearrangement: A mutation that juxtaposes nucleotides that are normally distant from each other, such as a chromosomal translocation, inversion or deletion.

Genotoxin: Chemical or other agent that modifies DNA and generates mutations.

Germline genome: An individual's genome as formed at the time of conception (fertilised oocyte). This genome is shared by all cells in the body.

Germline mutation: Mutations present in the germline genome. Sporadic mutations acquired in the germ cells of parents are also included in this category.

Germline variants: Variations in sequences or copy number of DNA segments observed between different individuals that are responsible for the phenotypic variation between people. Two unrelated individuals differ by approximately 3 million such variants.

Haplotype: A contiguous region of the genome containing a set of tightly linked genes that are usually inherited as a block.

Indel: A mutation that results in insertion or deletion of one or a few nucleotides to DNA.

Kataegis: Localised hypermutation of a region of the genome, thought to be mediated by APOBEC enzymes (from Greek καταιγίς = storm).

Linear evolution (of cancer): A form of clonal evolution of cancer that generates a single final clone of cancer cells which harbours all mutations that ever arose during its evolution.

Loss-of-function mutation: A mutation that leads to marked or complete loss of function of the coded protein.

Table 9.1 (*Continued*)

Loss-of-heterozygosity (LOH): A genetic modification leading to the loss of the maternally- or paternally-derived copy of a genomic region. This can happen as a result of deletion or uniparental disomy (uPD).

Methylation: Covalent addition of a methyl group to a DNA, RNA, protein or other molecule.

Missense mutation: A nucleotide substitution (e.g. G–T) that results in an amino acid change (e.g. valine to phenylalanine).

Mutational signature: A recurrent pattern of DNA mutations attributable to a particular type of mutagen or mutational process, characterised by certain nucleotide mutations in a specific 5′ and 3′ nucleotidic context.

Mutual exclusivity: The occurrence of two or more mutations in the same cancer type less often than would be expected by chance.

Next-generation sequencing (NGS): DNA sequencing using one of the methodologies developed since 2005 and which allow massively parallel sequencing of thousands or millions of fragments of DNA simultaneously.

Nonsense mutation: A nucleotide substitution that results in the generation of a stop codon (i.e. TAA, TGA or TAG).

Non-synonymous mutation: A mutation that alters the encoded amino acid sequence of a protein. These include missense, nonsense, splice site, gain of translation start, loss of translation stop and indel mutations.

Oncogene: A gene that confers growth advantage to a cell and contributes to its progression/transformation to cancer.

Opportunity hypothesis: A hypothesis proposing that the observed order of acquisition of somatic mutations during cancer evolution reflects the statistical likelihood that mutations are acquired in this order. This likelihood is determined by the earlier mutation influencing the opportunity for acquiring the next. Implicit in this hypothesis is that the reverse order can also be observed, albeit less often.

Passenger mutation: A mutation that does not give a selective growth advantage to its host cell.

Proto-oncogene: A gene that when activated by mutations, becomes an **oncogene** and imparts a growth advantage of its host cell.

Single nucleotide polymorphism (SNP): A DNA sequence variation occurring commonly within a population (e.g. 1%) in which a single nucleotide in the genome differs between individuals.

Somatic mutation: A mutation that occurs in any cell of the body after conception. Sometimes called *acquired mutation*.

Substitution (or nucleotide substitution): A DNA mutation leading to the replacement of a native nucleotide with another.

Splice sites: DNA sequences flanking exons which are important for mRNA splicing.

Subclonal mutation: A mutation that exists in only a subset of the neoplastic cells within a tumour.

Transition (mutation): Change of a nucleotide to another of the same group such as C > T (both pyrimidines) or G > A (both purines).

Transversion (mutation): Change of a nucleotide to another of the opposite group such as A > C (purine to pyrimidine).

Tumour suppressor gene: A gene that inhibits the development of cancer, commonly by limiting cell proliferation. These genes are frequently inactivated in cancer cells.

Uniparental disomy (uPD): A genetic modification leading to the loss of the maternally or paternally derived copy of a genomic region as a result of replacement of this sequence with the equivalent sequence derived from the other parent.

Untranslated region (UTR): An exonic region located before the start (5′ UTR) or after the stop (3′ UTR) codon of a gene and which does not code for any amino acids.

Whole genome sequencing: Sequencing of the entire sequence of an individual genome using germline DNA, tumour-derived DNA or DNA from another cellular source such as cell lines, single cells etc.

Whole exome sequencing: Sequencing of all the exons of all the genes in an individual genome (there are approximately 30,000 coding genes in a mammalian genome). As with whole genome sequencing this could be done using germline DNA, tumour-derived DNA or DNA from another cellular source.

X-linked gene: Any gene located on the X chromosome.

Next generation sequencing (NGS) technologies allow such analysis through parallel sequencing of thousands to millions of DNA molecules in a relatively short period of time. Compared to classical Sanger methodology this represents an increase in speed and capacity in excess of 1 billion-fold and a dramatic reduction in costs. NGS output is in a digital form, where each sequencing read, corresponding to an individual genomic DNA fragment, is captured separately. Reads are then mapped back to the reference genome using computational aligning algorithms, allowing the sequence of the *whole genome* to be analysed in one sequencing run. Alternatively, genomic regions of interest can be enriched for prior to sequencing, allowing the investigator to limit analysis to the coding portion of the genome (*whole exome sequencing*), or to specific parts of the genome such as the exons of a selected set of genes. Importantly, NGS is quantitative and can be used to discriminate between homozygous, heterozygous and subclonal events based on the number of DNA molecules supporting each variant (*allele burden*), see Chapter 8 for more details on next-generation sequencing and its application in this context.

The accumulation of somatic mutations is an inevitable consequence of the passage of time and this is true for any cell,

including the germ cells through which acquired mutations are passed to the offspring, generating diversity in the human and any other living species. However, mammalian genomes are afforded an extraordinary level of protection from mutations such that each nucleotide has only a 1 in 10^{10} chance of mutation per generation. Nevertheless, mutations do accumulate with time at a rate that is influenced by the abundance and nature of mutagens acting on cellular DNA and its proof-reading machinery.

A cancer cell, just like all the other cells in the body, is a direct descendant of the fertilised egg through a lineage of mitotic cell divisions. However, the genome of a cancer cell and indeed that of a normal cell harbour a set of somatic mutations acquired during the cell's own life and the life of its ancestors back to the fertilised egg (Figure 9.1). These are collectively termed **somatic mutations** to distinguish them from **germline mutations.** In the following paragraphs, we will describe examples of germline as well as somatic variants associated with cancer and discuss the processes leading to acquisition of somatic variants.

Classes of DNA mutations

The structure and sequence of genomic DNA can be altered in several ways by mutagens. The resulting changes belong broadly to one of two categories: those that change the nucleotide sequence at a local scale and those that affect large genomic regions such as parts of chromosomes or entire chromosomes (Figure 9.2). Mutations that change the nucleotide sequence of DNA are broadly classified as *substitutions* and *indels* (insertions or deletions). When substitutions affect coding exons, they can be *synonymous* (no change in the coded amino acid), *missense* (change of the coded amino acid to another amino acid) or *nonsense* (change of the coded amino acid to a stop codon). When indels affect coding exons, they can have similar effects. However, if the size of an indel is not a multiple of three nucleotides the resulting change is a *frameshift* mutation. Frameshift mutations lead to the translation of a novel aberrant sequence of amino acids terminating at an endogenous stop codon sequence.

Both substitutions and indels do occur outside coding exons where their effects are more difficult to decipher. When they affect splice junctions of exons, they can lead to reduced usage or complete exclusion of an exon from the final mRNA. When they occur elsewhere in the genome, they may not have a significant functional effect, but in some cases, they can affect gene regulatory sequences. Examples of such mutations having an oncogenic effect were recently described.

Mutations altering the copy number of regions of the genome are referred to as *amplifications* and *deletions*. They can affect part of a gene, several genes, large chromosome segments or whole chromosomes. With these mutations, the net gain or loss of genes has an oncogenic effect, although for large amplifications or deletions, it is often difficult to pinpoint which of the several genes involved are most important. *Chromosomal translocations* and *inversions* are a recurrent type of structural genomic rearrangement in haematological and other cancers. With these mutations, two regions of the genome situated far away from each other are juxtaposed as a result of breakages in their host chromosomes, followed by illegitimate fusions between them. These events can result in the formation of a fusion oncogene, such as *BCR::ABL* in the t(9;22) in CML (see Chapter 33) or lead to marked over-expression of a gene such as *CCND1* or *MYC* in the t(11;14) and t(8;14), respectively (Figure 9.3). In each of the latter two cases, the target gene is over-expressed as a result of coming under the control of the strong IGH promoter in mantle and Burkitt lymphomas, respectively. *Chromothripsis* is a very dramatic type of structural change affecting the genomes of some cancers. In chromothripsis, tens to hundreds of chromosomal rearrangements

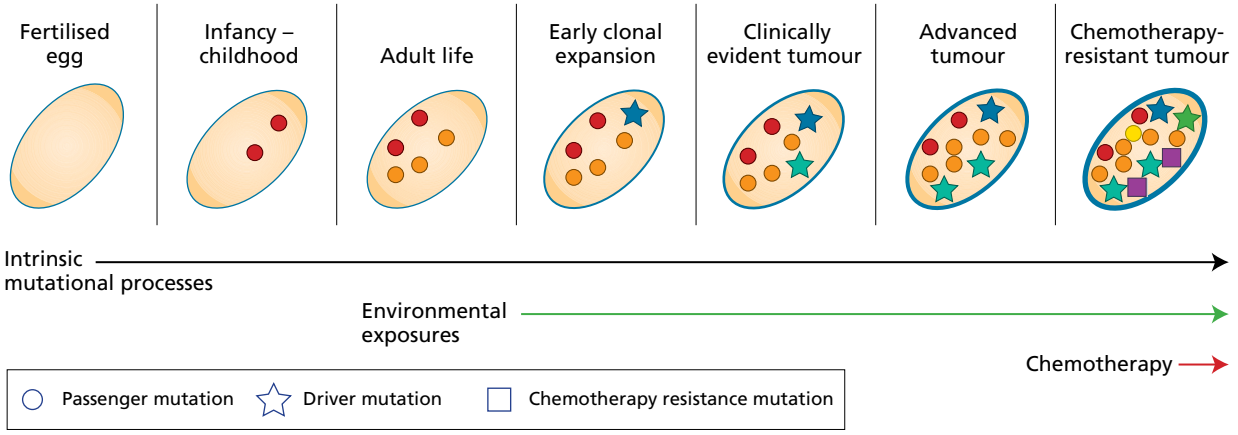

Figure 9.1 The sequence of somatic mutations acquired during cell divisions from the fertilised egg to a single chemoresistant cancer cell. The mutational processes causing these mutations are also highlighted. (Source: Modified from Stratton *et al.* 2009).

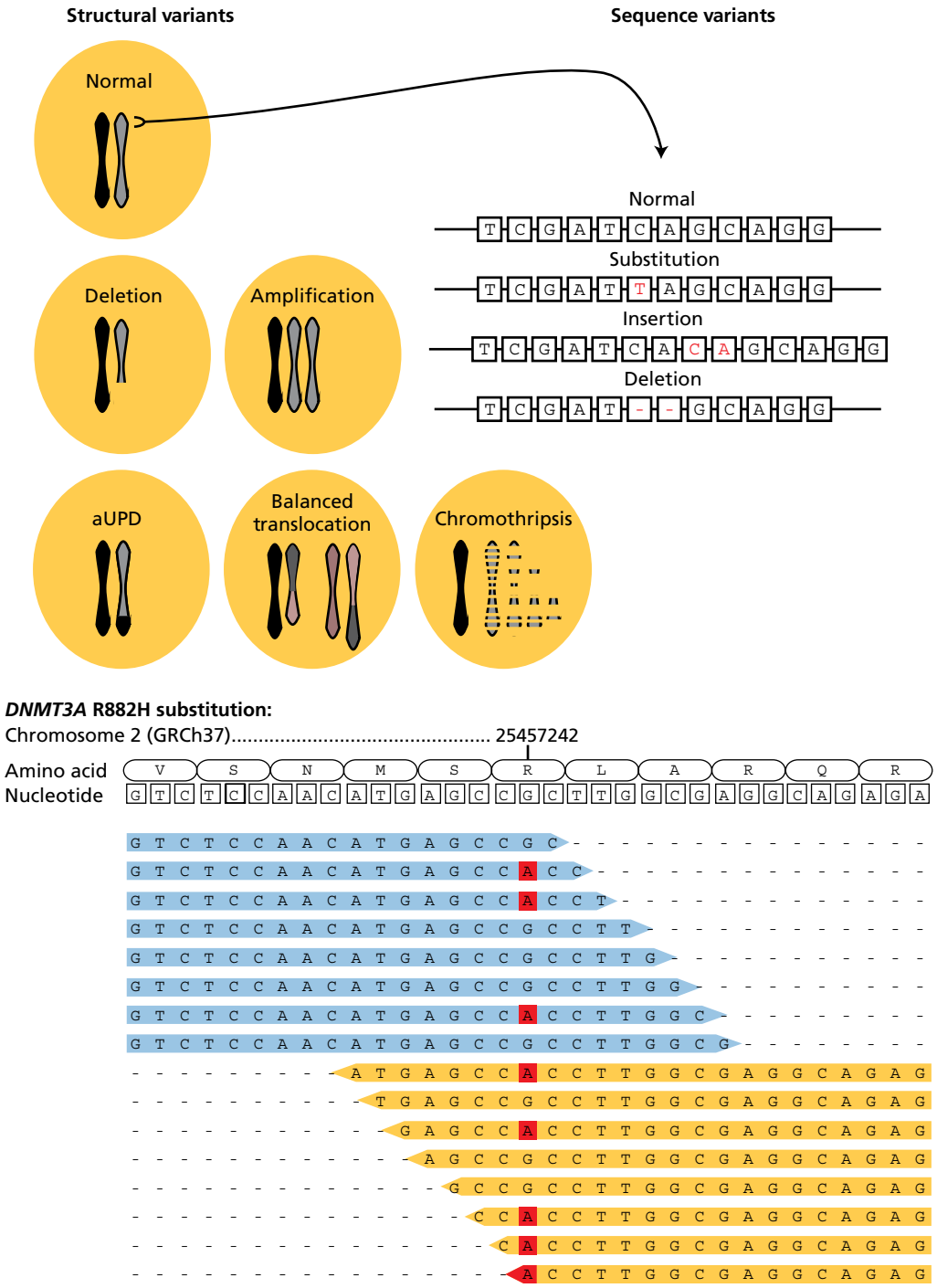

Figure 9.2 Different types of mutations and chromosomal rearrangements.

involving localised genomic regions can be acquired in an apparently single catastrophic event.

Finally, an important concept in cancer genomics is that of *loss-of-heterozygosity (LOH)*. As each of us inherits a maternal and a paternal allele for every gene or genomic region, we are heterozygous throughout our genome. Our heterozygosity can be verified using polymorphisms that distinguish the maternal and paternal alleles. In cancer, it is common to find that areas of the genome have 'lost their heterozygosity', as they only display one of the two alleles and this happens if one of the two alleles is lost. This can be due either to a deletion of that allele or its replacement by the other allele (which is now duplicated).

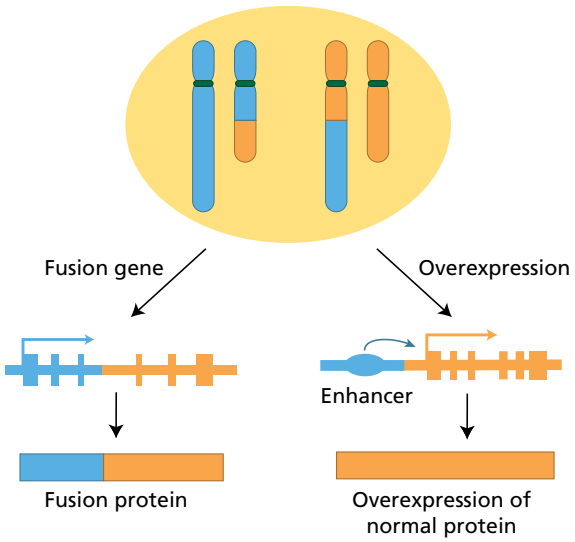

Figure 9.3 Common mechanisms of gene activation by chromosomal translocations: (i) formation of a chimaeric gene by fusion of the two genes spanning the chromosomal breakpoints and (ii) over-expression of an intact gene through the action of a potent enhancer translocated upstream.

The duplication of one allele and associated loss of the other is known as acquired *uniparental disomy (aUPD)* and is the result of mitotic recombination (or mitotic cross-over) between two homologous chromosomes. As there is no net loss of genetic material, aUPD is also known as *copy-neutral LOH*. Mitotic recombination occurs at a very low rate and the reason it appears a lot more frequently in cancer is because just like any other mutation, aUPD can give an additional growth advantage to the host cell, often by duplicating a previously acquired oncogenic mutation. For example, *FLT3* internal tandem duplications and the *JAK2V617F* mutation are commonly duplicated by aUPD.

Inherited predisposition to haematological cancers

Most haematological malignancies are sporadic and not attributable to identifiable heritable or environmental risk factors, with some important exceptions (Table 9.2). For example, the risk of developing blood neoplasms is higher in individuals with certain uncommon inherited syndromes. In most instances, what is inherited is a genetic predisposition to develop cancer(s) through transmission of genetic variants that are either oncogenic themselves or accelerate the acquisition of somatic mutations that cause overt cancer. As cancer is a multistep process, acquiring the first oncogenic variant through germline transmission significantly increases the chances of acquiring the full set of variants that generate a cancer during the person's lifetime, hence the increased risk of cancer in these individuals.

Table 9.2 Examples of risk factors for the development of leukaemia and lymphoma.

Germline syndromes
- Oncogenic variants
  - *RUNX1* (familial platelet disorder with propensity to myeloid malignancy)
  - *CEBPA* (rare cases of familial AML)
  - *DDX41* (Familial predisposition to myeloid neoplasms)
  - *NF1* (Type I neurofibromatosis)
  - *PTPN11* (Noonan's syndrome)
  - *CBL* (rare cases of JMML)
- Variants that increase genome instability
  - *BLM (Bloom's syndrome)*
  - *ATM (Ataxia Telangiectasia)*
  - *FA genes (Fanconi anaemia)*
  - *ALDH2 and ADH5 coinheritance (Aldehyde degradation deficiency syndrome)*
  - *ERCC6L2 (familial MDS and AML in children and young adults)*
- Predisposition alleles/haplotypes
  - *46/1 haplotype in PV/ET*
  - *Several alleles in multiple myeloma, e.g. rs603965 in t(11;14)*
  - *Several alleles in chronic lymphocytic leukaemia, e.g. rs13397985*

Endogenous mutagenic processes
- Spontaneous deamination of methylated cytosines at CpG islands
- Cytidine deaminases (AID, APOBEC)
- RAG-driven chromosomal breaks and translocations, e.g. in B-ALL carrying t(12;21)

Exogenous mutagenic processes
- Biological
  - *EBV infection (EBV-driven lymphoproliferative disorder)*
  - *HTLV-1 infection (adult T-cell leukaemia/lymphoma)*
  - *HHV-6 (Castleman's disease and lymphomas of serous cavities)*
- Chemical
  - *Benzene*
  - *Chemotherapeutic agents*
- Physical
  - *Ionising* radiation

Also, as germline mutations are present in all cells of the body, they can have developmental effects in several organs and tissues. For this reason, many of the common mutations acquired somatically in cancer are never seen as heritable mutations. For example, the acute myeloid leukaemia (AML)–associated *NPM1* mutations lead to early embryonic lethality in mice and unsurprisingly are never seen as germline mutations in humans.

In familial platelet disorder with propensity to myeloid malignancy (FPD/AML), heterozygous inheritance of a *RUNX1* oncogenic variant causes an autosomal dominant syndrome

characterised by mild to moderate thrombocytopenia, abnormalities of platelet function and a propensity to develop myelodysplasia (MDS) and/or AML. *RUNX1* is a transcription factor critical for the establishment of definitive haemopoiesis and a target of somatic mutations including *deletions*, *translocations*, *substitutions* and *indels* in leukaemia. Most familial *RUNX1* mutations in FPD/AML lead to premature stop codons or affect the Runt domain of the gene involved in DNA binding. The median incidence of myeloid malignancy in FPD/AML ranges between 20 and 65% between families, but even within the same family affected individuals may present with variable clinical severity and at varying ages. This highlights how *RUNX1* mutations are insufficient for carcinogenesis in isolation but require co-operating somatic mutations for cancer development. In fact, mutations affecting the other copy of *RUNX1* as well as unrelated chromosomal deletions and translocations are commonly seen at the time of leukaemia progression.

An increased risk of malignancy can also arise from inheritance of variants in genes that increase the acquisition of mutations required to transform a normal cell to a cancer cell. One such example is Bloom's syndrome, an autosomal recessive disorder characterised by predisposition to the development of haematological and solid cancers, along with a constellation of physical findings. In this disorder, biallelic mutations in the gene for the DNA helicase *BLM,* result in a widespread *genomic instability* and a highly increased rate of mitotic recombination leading to aUPD. This can lead to rapid homozygosity of oncogenic mutations and an increased growth advantage to the host cell. Fanconi anaemia is another autosomal recessive genetic disorder characterised by early onset bone marrow failure, leukaemia and solid cancers. Children with this disease inherit inactivating mutations in at least 23 genes in the Fanconi anaemia DNA repair pathway, leading to hypersensitivity to DNA crosslinking agents such as cisplatin. Reactive aldehydes, by-products of cellular metabolism, are a major endogenous source of DNA damage in Fanconi anaemia. Aldehydes are well known to cause DNA crosslinks, and cells deficient in the FA pathway are unable to repair aldehyde-induced DNA damage, ultimately leading to genomic instability and/or cell death. Interestingly, a bone marrow failure and leukaemia syndrome similar to Fanconi anaemia was discovered in several Japanese families with children who inherited inactivating mutations in two enzymes that detoxify aldehydes (ALDH2 and ADH5). Mouse models of this disease show that the loss of ALDH2 and ADH5 results in accumulation of endogenous formaldehyde, which acts as a potent *genotoxin* that disrupts haemopoiesis, and increases cancer risk.

As well as genetically well-defined syndromes such as the ones described above, a predisposition to cancer is also seen in some families with haematological malignancies, including CLL, myeloma, myeloproliferative neoplasms and lymphoma. The increase in risk is generally small and the genetic basis unclear, however *genome-wide association studies* (GWAS) have identified areas of the genome within which the genetic risk 'resides'.

An intriguing example of inherited risk comes from the observation that *JAK2* mutations, found in most cases of polycythaemia vera (PV) and also in other myeloproliferative neoplasms, are acquired preferentially within a particular *JAK2 haplotype* called 46/1. This haplotype, whose frequency in Europeans is approximately 25%, appears to 'invite' the acquisition of somatic *JAK2 V617F* mutations and gives 3–4 times increased risk of developing *JAK2 V617F*-positive myeloproliferative neoplasm. It is not clear what underlies this phenomenon, but one possibility is that acquisition of *JAK2 V617F* on this haplotype gives a greater growth advantage to the host cell than its acquisition on any other *JAK2* haplotype ('fertile ground hypothesis'). An alternative hypothesis is that the mutation occurs more frequently in this haplotype (hypermutability hypothesis).

Finally, another phenomenon of predisposition to a specific acquired mutation occurs in children with Down syndrome. In the first 3 years of life, up to 10% of these children develop transient myeloproliferative disorder (TMD), a disease that mimics AML, but typically resolves spontaneously within a few months (see Chapter 54). Almost all cases of TMD carry somatic mutations affecting the *X-linked* transcription factor GATA1, which drives the proliferation of leukaemic cells in TMD. The mutations disappear with the resolution of the TMD.

Acquired DNA mutations in haematological cancers

Although a lot can be learned from studying genetic susceptibility to cancer, neoplasms arise as a result of somatically acquired DNA mutations. The accumulation of somatic mutations is an inevitable consequence of the passage of time and this is true for all cells of all living organisms. However, mammalian genomes are afforded an extraordinary level of protection from mutations. Nevertheless, mutations do accumulate with time at a rate which is determined by the very small infidelity of the DNA replication and repair machinery and by the degree of exposure to endogenous or exogenous mutagens. Therefore, each somatic cell and by extension each cancer cell harbours a variable number of somatic mutations, which accrued during its lifetime and that of its ancestors from the fertilised egg onwards (Figure 9.1). This cargo of mutations in the cancer cell genome can be generated through a number of different mutational processes. The number and the nature of the mutations can provide important pathogenic information and in some cases identify the responsible mutagen and the extent of its impact on the genome. For example, it is evident that the total number of mutations identified in the genomes of different forms of cancer varies widely and reflects the type and length of exposure to different

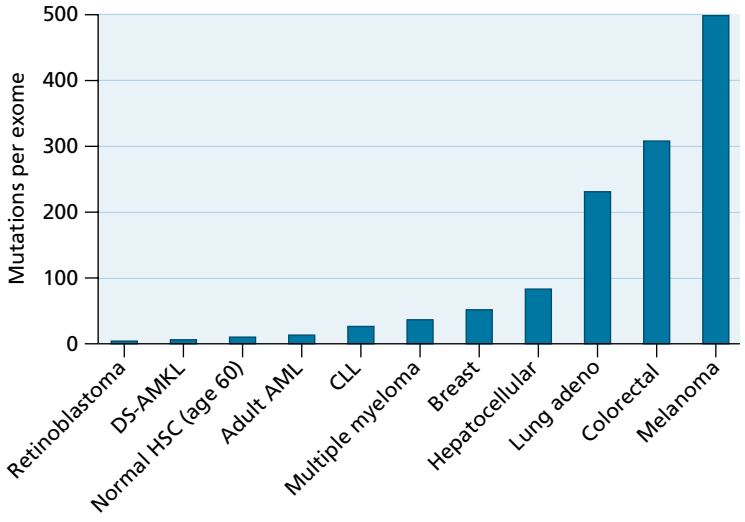

Figure 9.4 Median number of mutations per exome in various haematological and solid cancers.

mutagens (Figure 9.4). In addition, by sequencing multiple pre-cancerous and cancer genomes, we can model the rate of mutation accruing in cells. This has revealed that the earliest cancer-associated mutations can be acquired within the first two decades of life. Also, studies of the particular type of change in DNA sequence can give important information about the mutagens and processes involved. More than 30 such *mutational signatures* have now been described and for many of these, the responsible biological processes or mutagens have been identified. Below we discuss some of these processes.

Endogenous processes

A specific type of DNA mutation that affects all cells is the spontaneous deamination of methylated cytosines primarily at CpG islands which, if not corrected by editing enzymes, leads to a C > T *transition*. This process causes mutations at a very slow, but constant, rate and is thought to account for most of the spontaneous background mutation rate and for the under-representation of CpG dinucleotides in mammalian genomes. It is thought to play a role in the evolution of species and can lead to cancer development if it causes oncogenic mutations. In fact, it is the only recognisable mutational process in most AML genomes.

In addition to spontaneous deamination, enzymatic deamination of cytosines in DNA by the enzyme family APOBECs (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) and AID (activation-induced deaminase) can create C > T *transition* and C > G/A *transversion* mutations. APOBECs and AID can deaminate cytosine in both RNA and DNA substrates to play diverse physiological roles. For example, APOBEC1 edits the apolipoprotein B mRNA to introduce a premature stop codon required for the synthesis of shorter forms of apolipoprotein B. The APOBEC3 complex plays a role in anti-viral immunity through the targeted hypermutation of viral DNA genomes such as the cDNA intermediate of the HIV. AID deaminates cytosine in B lymphocyte immunoglobulin genes to initiate secondary antibody diversification via somatic hypermutation and class-switch recombination. Both APOBECs and AID have substrate preference for cytosines flanked by specific nucleotides. In the case of APOBECs, these enzymes preferentially deaminate cytosine in the TpCpN trinucleotide sequence (N represent any nucleotide). For AID, the preferred substrate is RpCpW (where R = A/G, W = A/T). Through the analysis of these mutational signatures, it has been observed that many mutations at cytosines are attributed to the off-target mutational activity of APOBECs/AID in haematological cancers, including B-ALL, CLL, MM and DLBCL. In addition, the AID/APOBEC mutation signatures are often associated with local hypermutation of regions of the genome, a phenomenon known as *kataegis*, representing localised mutation 'storms' at different genomic regions. Ectopic action of AID has been detected at a number of secondary sites, some of which have been implicated in the pathogenesis of CLL and DLBCL (e.g. *BCL-6* mutations and *IGH* translocations).

Another group of DNA editing enzymes, the RAG endonucleases *RAG1* and *RAG2,* mediate the physiological V(D)J recombination process during B-cell development. In cases of B-ALL carrying t(12;21) and expressing the chimeric *ETV6::RUNX1* fusion protein, the RAG enzymes can be aberrantly activated to drive a mutational process characterised by deletions scattered across the genome at regions flanked by recombination signal sequence motifs. These deletions can be oncogenic and collaborate with *ETV6::RUNX1* to drive B-ALL.

There are almost certainly other endogenous processes that have yet to be discovered. For example, through analysis of mutational signatures, we know that signature 5 is prevalent in leukaemia, and in many solid cancers, but the mutagenic process that underpins this signature remains unknown.

Exogenous processes

The best-known mutagen, tobacco smoke, causes mutations with a particular signature which abound in the genomes of lung and some other epithelial cancers from smokers. So far, no evidence of this signature has been described in haematological cancers and the same can be said for UV-induced mutations. However, exposure to a number of exogenous biological, chemical and physical agents can lead to haematological cancers. One of the best-studied examples is the correlation between Epstein–Barr virus (EBV) infection and development of cancers, including lymphomas. Asymptomatic EBV infection can be demonstrated in >90% of the world population, in whom the virus can establish a latent infection in B-lymphocytes. The latent virus expresses a set of genes/antigens to maintain the viral genome and to successfully evade host immune surveillance. The same antigens have the ability to manipulate several cellular pathways and promote B-cell proliferation. This proliferation is normally kept in check by EBV-specific T lymphocytes that continuously survey and suppress infected B cells. However, in some highly immunosuppressed individuals, such as bone marrow or solid organ transplant recipients, this suppression fails and an EBV-driven B-cell lymphoma develops (post-transplant lymphoproliferative disorder). EBV is also implicated in the pathogenesis of Burkitt lymphoma and Hodgkin lymphoma, although the mechanism leading to neoplastic transformation is less understood. Similarly, infection with the human T-lymphotropic virus type 1 can lead to development of adult T-cell leukaemia/lymphoma, whilst HIV infection significantly increases the risk of B-cell lymphomas through reduction of anti-tumour immuno-surveillance, a risk that can be mitigated by combination antiretroviral therapy.

Amongst oncogenic chemical and physical agents in haematology, exposure to benzene, ionising radiation, alkylating agents and other chemotherapeutic agents have been clearly linked to the development of leukaemia and lymphoma with variable latency. A particular example is that of the association between exposure to topoisomerase inhibitors, such as etoposide and anthracyclines, and the development of acute leukaemia associated with chromosomal translocations involving the mixed lineage leukaemia (*MLL*) gene on chromosome 11q23. Topoisomerase II (Topo II) creates double-stranded breaks during execution of its function of relaxing overwound DNA, and it appears that Topo II inhibitors may stabilise complexes that are formed between the enzyme and free DNA ends, thus increasing the likelihood that those ends might participate in a translocation. The possibility has been raised that naturally occurring Topo II inhibitors have a causative role in some cases of leukaemia, but there is no good evidence to support this.

From genotype to phenotype

Regardless of how mutations are generated, most of them will not impart a growth advantage and are deemed to be 'passengers'. In most cancers only a minority of mutations are 'drivers', a term used to signify mutations that impart oncogenic properties to the host cell(s) and drive cancer growth; either by activating *oncogenes* or inactivating *tumour suppressor genes*. While a definitive categorisation of any mutation as a driver would require functional evidence that the variant can actually cause cancer, this is impractical for the ever-increasing number of variants that are identified by NGS studies. Instead, the 'driver' label is commonly given to mutations that are seen in any cancer type more often than would be expected by chance. Implicit in this working definition is the fact that it relies on some statistical calculation of 'chance' and is therefore prone to some error. In other words, some of the less frequently observed true drivers may not make the cut, whilst some passengers may fortuitously do so.

Another important concept to highlight here relates to the fact that each cancer has several driver mutations that work together to endow the cancer cells with their neoplastic phenotype. When studying these mutations, what becomes clear is that certain mutations co-occur regularly in the same cancer (e.g. *NPM1* and *FLT3* mutations in AML), whilst others almost never co-occur (e.g. *JAK2* and *CALR* mutations in MPD). When these observations are combined with what we know about the function of individual mutations, it becomes apparent that mutations that co-occur more often than expected by chance (*co-occurrence*), complement each other in transforming the host cell. By contrast, mutations with similar function do not co-occur or do so less often than expected by chance (*mutual exclusivity*), as only one or the other is sufficient to cause a specific effect or subvert a particular pathway, or in the case of spliceosome mutations, combinations of mutation might result in synthetic lethality.

Below we discuss some important examples of driver mutations from different haematological malignancies and highlight some of the better-understood genetic pathways subverted in these cancers.

NOTCH-signalling and lymphoid malignancies

NOTCH1 is a cell surface receptor with a central role in T-lymphopoiesis. It regulates interactions between adjacent cells and is activated by ligand binding, which triggers heterodimerisation of the receptor and proteolytic cleavage of an intracellular peptide (ICN1 = intracellular domain of NOTCH1) that migrates to the nucleus, where it affects DNA transcription. In the bone marrow, NOTCH1 signalling is required to promote the generation of T-lymphoid precursors from early haemopoietic progenitors, at the expense of B-lymphopoiesis. T-cell precursors subsequently migrate to the thymus, where NOTCH1 signalling is essential for survival and differentiation of T-cells with a successfully rearranged T-cell receptor. Furthermore, NOTCH1 is required for T-cell proliferation in the presence of antigen-presenting cells in peripheral tissues.

Given its physiological roles above, it is not surprising that in the multistep process leading to T-cell lymphoblastic leukaemia (T-ALL), constitutive activation of NOTCH1 signalling is one of the most prominent genetic events. It is present in 60% of T-ALL cases, and it invariably results in constitutive activation of ICN1 and its transcriptional activity. Interestingly, deregulated ICN1 activity can be achieved through different molecular mechanisms, all of which represent subversions of the physiological pathway (Figure 9.5). Point mutations affecting the heterodimerisation domain of NOTCH1 result in ligand-independent activation, while truncating mutations of the C-terminal regulatory PEST domain result in increased half-life of ICN1. Rarely, *NOTCH1* is involved in the chromosomal translocation t(7;9), which results in the expression of a truncated and constitutively active form of the protein. Finally, loss-of-function mutations of *FBXW7*, an E3 ubiquitin protein ligase that targets ICN1 for degradation, similarly result in its constitutive activation. Studies have shown that ICN1 over-expression causes leukaemia by altering the transcriptional profile of the cell and in particular by activating the oncogene *MYC*. Amongst co-operating recurrent events are deletions of the *CDKN2A* tumour suppressor locus, encoding a negative regulator of the cell cycle and a positive regulator of p53, present in up to 70% of T-ALL. In addition, T-ALLs characteristically show translocations and aberrant expression of transcription factor oncogenes, and deletions of other tumour suppressor genes. Clearly, different T-ALL cases will display different arrays of such genetic lesions; however, it appears that T-ALL cells rely on NOTCH1 signalling independent of its mutational status, making the pathway an attractive therapeutic target.

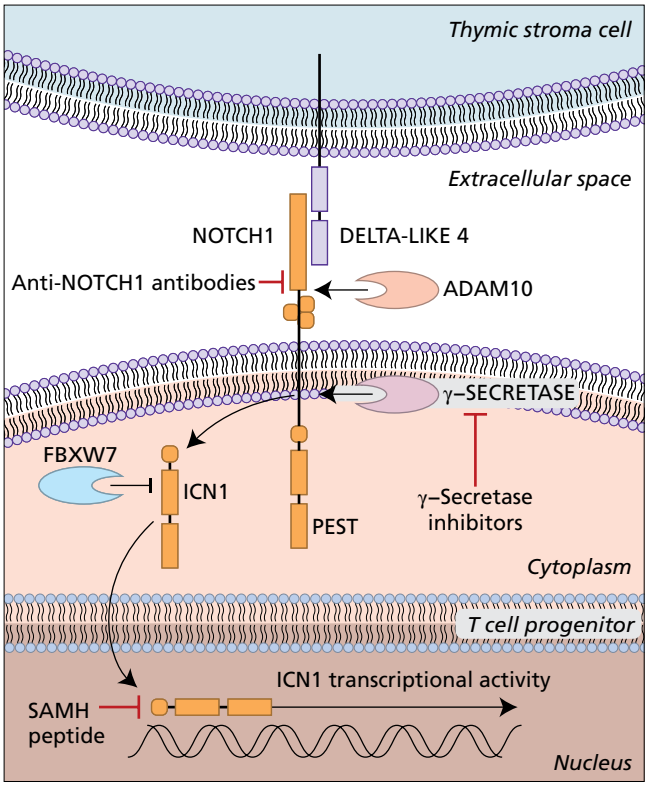

Figure 9.5 Schematic representation of NOTCH1 signalling and possible inhibitory strategies. NOTCH1 undergoes a double proteolytic cleavage upon interaction with the delta-like 4 ligand. The first is by the ADAM10 metalloprotease and can be inhibited by anti-NOTCH antibodies. The second cleavage is by the gamma-secretase complex, which can be targeted by small molecules. This double cleavage results in the release of the intracellular domain of NOTCH1 (ICN1) which activates transcription of target genes in the nucleus. FBXW7 antagonises this activity by targeting activated NOTCH1 to the proteasome. Lastly, small SAMH1 peptides disrupt the NOTCH1 nuclear transcriptional complex.

The well-defined role of NOTCH1 in T-cell development explains the relative specificity of these mutations to T-ALL. However, recently NOTCH1 PEST domain mutations were identified in approximately 10% of CLL, where they constitute a poor prognostic indicator. In most cases, *NOTCH1* mutations occur in the subgroup of CLL with unmutated IGHV genes and in cases with trisomy of chromosome 12 as the sole cytogenetic abnormality. Unlike T-ALL, *NOTCH1* mutations in CLL are almost exclusively frameshift or nonsense events, clustering within a hotspot in exon 34 of the gene and generating a truncated protein. The identification of *NOTCH1* mutations in CLL may be somewhat surprising, given the role of this gene in T-cell development; however, NOTCH1 signalling has a well-defined role in promoting terminal differentiation of mature B-lymphocytes to antibody-secreting cells. The theme of cancer mutations targeting genes involved in physiological pathways important to different types of cells in the body is very common in both haematological and solid tumours.

Epigenetics and leukaemia

The regulation of transcription is the fundamental regulatory node for controlling gene expression. The process requires physical access of specialised proteins to genomic DNA in order to read and transcribe the code into RNA. However, every human cell contains about 2 m of linear genetic information, which is packaged into a nucleus with a 6 µm diameter. This packaging is achieved by 'wrapping' genomic DNA around histones, a family of ultra-highly conserved nuclear proteins. This moulds genomic DNA into a beads-on-string configuration, which is then further folded and tightly compacted into a macromolecular complex known as chromatin. This makes access to DNA by transcriptional machinery very challenging, but also turns histones into the custodians of such access; a role which they conduct primarily through their N-terminal protein 'tails'. The process is controlled by

different post-translational modifications of these histones, including methylation, acetylation and phosphorylation, which can lead to increased or decreased access to DNA and by extension to a higher or lower level of gene transcription, respectively. Additionally, changes to the DNA itself have a further role in controlling gene expression. The area of biology concerned with these phenomena is known as epigenetics and corruption of epigenetic processes is a frequent event in leukaemogenesis.

Amongst the better-understood examples of leukaemogenic mutations operating through their aberrant effects on chromatin are those affecting the *MLL* gene. MLL is a lysine-specific methyltransferase which normally methylates specific lysine residues in histone tails to alter chromatin accessibility and increase gene expression. In the haemopoietic system, the MLL protein activates transcription of posterior *HOXA* genes (*HOXA7-A10*), which promote the self-renewal of HSCs and immature progenitors. In normal haemopoiesis, their expression is maintained by MLL in the HSC/immature progenitor compartments and diminishes as cells differentiate. However, *MLL* is a recurrent target of chromosomal translocations, which lead to an aberrant fusion of this gene to one of more than 80 gene partners. MLL-fusion proteins hijack the function of MLL and misdirect it to histone lysine residues, which are not normally targeted by the wild-type MLL. For example, the *MLL::AF9* fusion protein was shown to be co-recruited with normal MLL (expressed from the intact MLL allele) to the *HOXA9* gene locus, where the two proteins methylate different lysines on local histones to 'open' the chromatin and increase *HOXA9* mRNA expression (Figure 9.6). This results in aberrant self-renewal of haemopoietic progenitors, leading to leukaemia.

Recent developments in this area have identified that BET (bromodomain and extraterminal) proteins are important for recruiting MLL fusion proteins to chromatin. Inhibitors of the interaction between MLL fusion proteins and BRD4, a BET family member, have shown potent antileukaemic activity against MLL-fusion leukaemias and have recently entered clinical trials.

As well as its effects on chromatin, leukaemogenic mutations can alter the normal processes involved in the other important set of epigenetic events, namely modifications of DNA itself. The most important DNA modifications are those affecting cytosine residues, which can be methylated by DNA methyltransferases such as DNMT3A and DNMT3B to form methylcytosine, a residue that is generally inhibitory to gene transcription. Additionally, cytosine methylation is an important process for suppressing the expression of the large numbers of genomic parasites found in mammalian genomes, such as transposons and endogenous retroviruses. However, cytosines at particular parts of the genome rich in cytosine-guanine dinucleotides and known as CpG islands, are not always methylated. CpG islands are frequently located at the promoter regions of genes and methylation of cytosines in these islands is associated with reduced gene expression. In fact, unmethylated CpG cytosines are frequently found in regions of 'open' chromatin where histones allow the transcription machinery access to the genomic DNA.

So how do cytosines become unmethylated? With the exception of germ cells, the process was poorly understood until recently. Instead of demethylation, methylcytosines can undergo hydroxylation to hydroxymethylcytosines by enzymes known as TET dioxygenases; namely TET1, TET2 and TET3. These enzymes can further modify hydroxy-methylcytosine to formyl- and then carboxylcytosine, which can then be converted back to a cytosine. Importantly, the hydroxyl group for the initial hydroxylation by TETs comes from α-ketoglutarate produced by the action of isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2) as an intermediate in the tricarboxylic acid (Krebs) cycle (Figure 9.7).

A number of key genes involved in the above processes are recurrently mutated in myeloid and less often in some lymphoid malignancies. In particular, *DNMT3A* mutations are found in 30%, *TET2* in 10%, whilst *IDH1* and *IDH2* are each mutated in 7–8% of cases of AML. Additionally, all of these genes are mutated in other myeloid neoplasms including MDS, MPN and CMML. Both *DNMT3A* and *TET2* are targeted by nonsense or other loss-of-function mutations that can be homozygous or heterozygous. However, the most frequent *DNMT3A* mutation is a missense mutation leading to substitution of arginine 882 with histidine (R882H) or

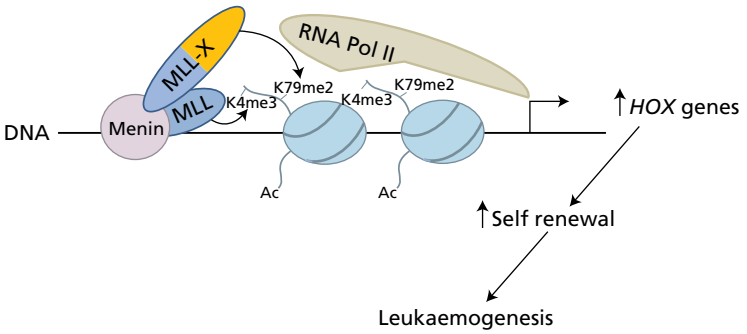

Figure 9.6 Functional consequences of disrupted MLL activity by an MLL fusion protein (MLL-X). The fusion protein acts in a dominant manner recruiting the normal MLL protein to lysine residues (K4 and K79) of histones that are not normally targeted by MLL and whose methylation promotes active and aberrant transcription of the downstream genes (such as *HOXA* genes).

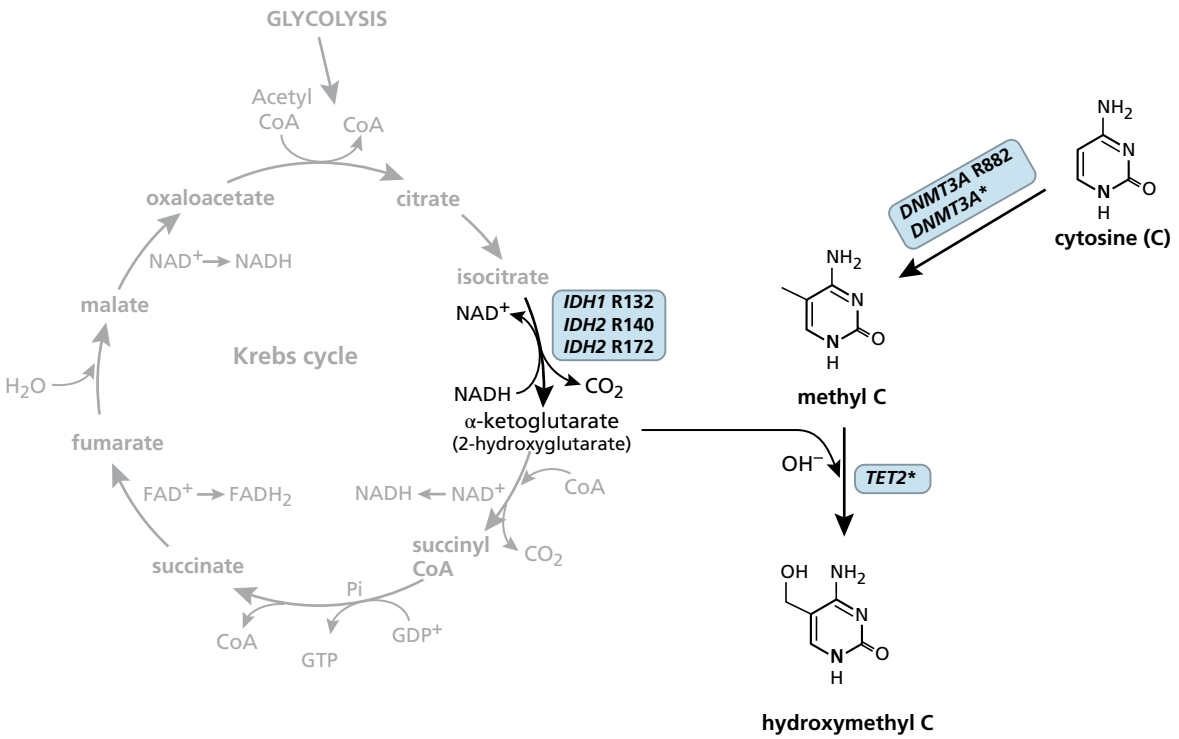

Figure 9.7 Enzymes involved in DNA methylation whose mutations are implicated in myeloid disorders. The metabolic reaction catalysed by each enzyme is indicated along with the most frequent leukaemogenic mutations (blue boxes + see text). The oncometabolite 2-hydroxyglutarate is produced, instead of α-ketoglutarate, by the action of mutant *IDH1* or *IDH2*. 2-hydroxyglutarate inhibits *TET* dioxygenases (including *TET2*) and other epigenetic enzymes.

cysteine (R882C) and less commonly serine (R882S) or phenylalanine (R882P). These substitutions are almost always heterozygous, but they are thought to have a dominant negative effect, i.e. as well as lacking methylase activity themselves, they act to inhibit the methylase function of the remaining normal *DNMT3A* allele, although they may also have other effects. Mutations affecting *IDH1* and *IDH2* have the effect of disrupting the enzymatic function of TET proteins by generating the 'oncometabolite' 2-hydroxyglutarate (2-HG) instead of α-ketoglutarate. There is now good evidence that 2-HG mediates the oncogenic properties of these mutations, by inhibiting the function of TETs and also that of a number of chromatin-modifying enzymes such as histone demethylases. In keeping with this, *IDH1, IDH2* and *TET2* mutations do not occur together in the same AML, supporting the premise that they influence similar genetic pathways (see mutual exclusivity and co-occurrence earlier in this chapter). Studies to understand the molecular consequences of *DNMT3A, TET2* and *IDH* mutations have demonstrated that their effects on DNA methylation are genome-wide rather than being specific to leukaemia genes. In other words, it is probable that they change gene expression of some genes that promote leukaemia and others that do not. However, their net effects are pro-leukaemic.

Mutations affecting *IDH1* and *IDH2* are *gain-of-function* changes, which in contrast to loss-of-function mutations are usually more amenable to targeting by new therapies. The specific mutations affecting codons *IDH1* R132, *IDH2* R140 and *IDH2* R172 (Figure 9.7) are not only seen commonly in myeloid malignancies but also occur in non-haemopoietic cancers such as glioblastoma and cholangiocarcinoma. *IDH1* and *IDH2* inhibitors are now in clinical trials and showing significant promise against AMLs carrying these mutations.

Tyrosine kinases and myeloproliferation

Tyrosine kinases are a large diverse family of enzymes that catalyse the transfer of phosphate groups from ATP onto tyrosine residues on target proteins (see also Chapters 33 and 35). These enzymes can exist within cell surface receptors, known as receptor tyrosine kinases (RTKs) e.g. KIT, PDGFR, FGFR or as free cytoplasmic enzymes e.g. *JAK2* and ABL1. Tyrosine kinases play essential roles in cell signalling, relaying both intracellular and extracellular signals to regulate core functions such as proliferation, transcription, translation, energy metabolism and differentiation. In haematopoiesis, tyrosine kinase signalling pathways underpin the regulation of haemopoietic stem and progenitor cells for proliferation and differentiation. It is thus

not unsurprising that tyrosine kinases and their regulators are targets of leukaemia driver mutations.

Cell signalling via RTK begins with the binding of a ligand to the extracellular surface of the receptor. Ligand binding promotes receptor dimerisation, which in turn stimulates autophosphorylation of specific tyrosine residues within the intracellular part of the protein. The dimerisation and associated increase in kinase activity result in recruitment of effector molecules and activation of downstream signalling pathways. Many cytoplasmic tyrosine kinases are also thought to be activated by phosphorylation and dimerisation. Driver mutations in tyrosine kinases result in constitutive kinase activity without the need for prior activation. Individual myeloproliferative disorders are associated with activation of different tyrosine kinases, with specific correlation between gene mutations and the resulting neoplastic phenotype. For example, the kinase activity of *JAK2* is activated by point mutations (e.g. Val617Phe) in almost all cases of polycythaemia vera and 50% of essential thrombocythaemia. Mutations in the thrombopoietin receptor (MPL) or in Calreticulin (*CALR*) are alternative [mutually exclusive] mutations which can also active *JAK2* signalling) see Chapter 35 for more details on this and other aspects of MPN. Point mutations in *KIT* (Asp816Val) are present in 90% of cases of systemic mastocytosis. As a consequence of these changes, the corresponding signalling pathway is activated and this provides the transformed cell with a proliferative or survival advantage.

Tyrosine kinases can also be activated by translocations, like in CML where the *BCR* gene becomes fused in-frame with the *ABL1* tyrosine kinase gene as a consequence of the Ph translocation (Figure 9.8). This usually results in the formation of a fusion protein of 210 kDa (hence p210 *BCR::ABL*), see Chapter 33 for more details on this and other aspects of CML. For example, activation of the RAS pathway is thought to contribute to the increased cell division and proliferation seen in CML cells, altered interactions with the actin cytoskeleton may underlie the diminished adhesion to inhibitory bone marrow stromal cells and STAT5-dependent upregulation of BCL-XL is implicated in the reduced apoptosis of CML progenitors.

As shown in Figure 9.8, two other versions of the *BCR::ABL* protein exist in which the breakpoint occurs in different introns of the *BCR* gene than those usually seen in CML. The smaller protein, p190 *BCR::ABL*, is found in Ph-positive ALL and has a higher level of constitutive tyrosine kinase activity than the p210 *BCR::ABL* protein. This is thought to contribute to the more aggressive behaviour of the ALL. A p230 *BCR::ABL* isoform is found rarely in patients with CML. In some cases, it is associated with a morphological picture resembling chronic neutrophilic leukaemia and possibly a lower rate of acute transformation. These variants illustrate how small structural differences in a fusion protein can cause significant differences in disease phenotype.

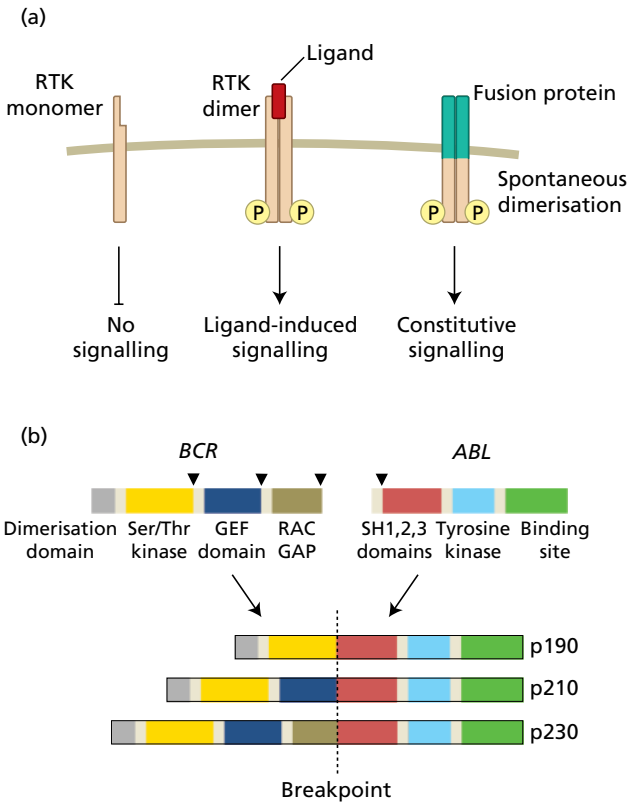

Figure 9.8 Fusion tyrosine kinases. (a) In normal cells, receptor tyrosine kinases (RTKs) exist as inactive monomers which, upon ligand binding, become activated by dimerisation and autophosphorylation. By contrast fusion tyrosine kinases dimerise spontaneously and are constitutively active. (b) Different forms of *BCR::ABL1* fusion genes contain varying lengths of the *BCR* gene and result in different molecular effects (see text). Unlike the examples in (a) that depict transmembrane *RTK*s, *ABL1* and *BCR::ABL1* are intracellular TKs.

Receptor tyrosine kinases such as *PDGFRA*, *PDGFRB* and *FGFR1* can participate in fusion genes found in eosinophilic myeloproliferative disorders. A remarkable feature of these syndromes is the large number of fusion partners described for each of these kinases, with more than 10 partners currently known for *FGFR1*, more than 5 for *PDGFRA* and more than 20 for *PDGFRB*. As with *BCR* in *BCR::ABL*, most of these partners harbour dimerisation domains that facilitate constitutive activation of the tyrosine kinase. However, reflecting their molecular heterogeneity, these disorders exhibit significant clinical diversity. For example, cases associated with the *FIP1L1::PDGFRA* fusion exhibit marked eosinophilia, while those associated with *ETV6::PDGFRB* often show dysplastic features and a lesser degree of eosinophilia. Additionally, cases involving *FGFR1*, collectively known as the *8p 11 myeloproliferative syndrome,* are

usually associated with a co-existing non-Hodgkin lymphoma. Notably, cases associated with rearrangements of the *PDGFR* genes respond well to imatinib, while those associated with *FGFR1* fusions do not. Newly developed FGFR inhibitors are currently being tested against the latter.

GM-CSF and RAS signalling in juvenile myelomonocytic leukaemia

Juvenile myelomonocytic leukaemia (JMML) is a rare disorder of childhood characterised by a marked expansion of both myeloid and monocytic lineages, with varying degrees of dysplasia and exquisite sensitivity to granulocyte–macrophage colony-stimulating factor (GM-CSF) in colony assays. These features reflect the fact that components of the GM-CSF receptor signalling and its key downstream effector, the RAS pathway, are frequently mutated in JMML (Figure 9.9).

The *RAS* genes (*KRAS, NRAS* and *HRAS*) code for small GTPases that operate as molecular switches, which are 'on' when bound to GTP and 'off' when the GTP is hydrolysed to GDP. Ras has only weak GTPase activity and in order to hydrolyse GTP and turn itself 'off' it requires activation by a GTPase activating protein (Ras-GAP). Once GTP is hydrolysed to GDP, Ras re-activation requires that GDP is exchanged for GTP. The latter process is catalysed by guanine nucleotide exchange factors (GEFs), which release GDP and allow Ras to bind the more abundant GTP. Once active, Ras stimulates cell proliferation by activating a number of cellular pathways, including mitogen-activated protein kinase (MAPK), PI3 kinase (PI3K) and phospholipase C epsilon (PLCε).

Mutations in the *NRAS* and *KRAS* genes are seen in many solid cancers, but also in several haematological malignancies, including AML, MDS, chronic myelomonocytic leukaemia (CMML), myeloma and others. Analyses of samples from patients with JMML show point mutations in *NRAS* and *KRAS* in approximately 25% of cases. The point mutations cause single amino acid substitutions, which result in constitutively active forms of the protein and thus mimic markedly amplified signalling from the GM-CSF receptor. The neurofibromin (*NF1*) gene has Ras-GAP activity and is therefore a Ras inhibitor. Interestingly, germline inactivating mutations in the *NF1* gene cause neurofibromatosis type 1, an autosomal dominant disorder characterised by protean clinical features, including increased risk of developing JMML and other tumours. Patients who have both neurofibromatosis type 1 and JMML have a high frequency (60%) of somatic inactivating mutations of the second *NF1* allele in the leukaemic cells. Furthermore, inactivating *NF1* mutations are found in 15% of sporadic cases of JMML without the clinical syndrome of neurofibromatosis. It appears, therefore, that *NF1* acts as a classical tumour suppressor gene, where two-step inactivation of both alleles allows unchecked signalling through the RAS pathway. A similar story holds true for the gene *PTPN11*, which encodes the tyrosine phosphatase SHP2. Germline mutations of this gene can cause the developmental disorder Noonan's syndrome, which is associated with an increased incidence of JMML. Up to 35% of non-syndromic patients with JMML have somatic mutations of *PTPN11* in their leukaemic cells. The mutations all cause amino acid substitutions clustering in the N-terminal SH2 domain of the protein, which is thought to result in enhanced phosphatase activity and activation of the downstream RAS pathway, giving the host cells a growth advantage over their normal counterparts.

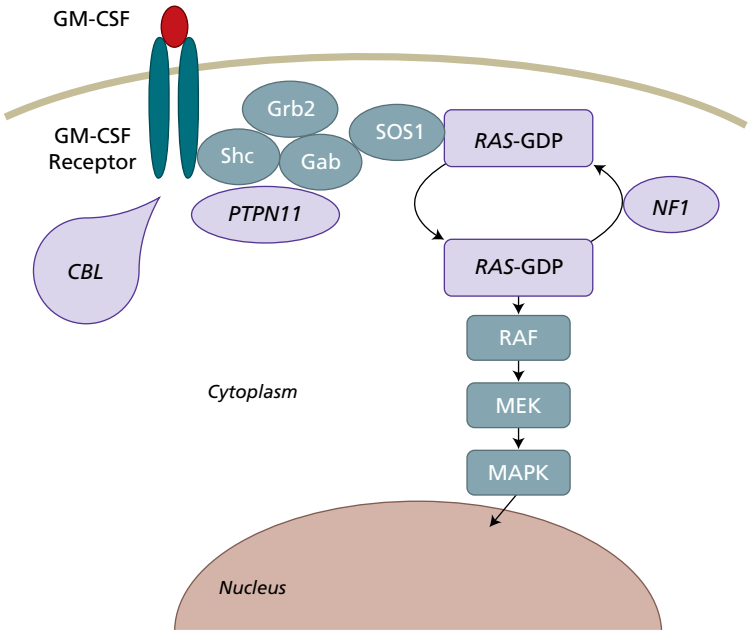

Figure 9.9 The RAS pathway and juvenile myelomonocytic leukaemia (JMML). The Ras pathway mediates many of the downstream effects of cytokine receptor activation. In most cases of JMML, the pathway is activated by mutations in one of four genes depicted in black text, which are involved in signalling downstream of the GM-CSF receptor: *RAS* (25% of cases), *PTPN11* (also known as SHP2, 35% of cases), *NF1* (15% of cases) and *CBL* (10–15% of cases). All of these mutations lead to *RAS* activation, which signals to a number of downstream effectors, including the RAF-MEK-MAPK pathway, to alter gene transcription and promote cell proliferation.

Most recently, mutations in the gene CBL were identified in 10–15% of JMML cases. Their identification followed the observation that some JMML cases exhibited aUPD of the long arm of chromosome 11. Further studies showed that some of these patients were born with germline heterozygous loss-of-function mutations in CBL, which became homozygous by duplicating the part of chromosome 11 carrying the mutation. CBL is an E3 ubiquitin ligase that controls proliferative signalling networks by downregulating the growth factor receptor signalling cascades in various cell types. JMML-associated mutations thus lead to complete loss of E3 ligase activity and increased GM-CSF receptor kinase activity and downstream signalling.

Thus, the common theme underlying most JMML is enhanced GM-CSF receptor pathway signalling, and this can be achieved by activating mutations of a positive regulator (such as *RAS* or *PTPN11*) or inactivating mutations of a negative regulator (*NF1* or *CBL*). Not surprisingly, mutations in *RAS*, *NF1*, *PTPN11* or *CBL* are mutually exclusive, suggesting that mutation of just one component is sufficient to activate the signalling pathway and that additional mutations in the pathway offer little additional advantage. Other mutations that have been reported to occur less frequently in JMML target the genes include *ASXL1, FLT3, SETBP1* and *JAK3*, with the last two conferring a poorer prognosis.

Other Ras pathway mutations in haematological malignancies

The Ras protein superfamily comprises more than 100 related small GTPases with diverse functions, including the Ras, Rho, Rab, Rap and Arf subfamilies. Mutations in *HRAS*, discovered in 1982, were the first example of a human-transforming oncogene. The closely related *KRAS* and *NRAS* were also found to be mutated in a plethora of different solid and haematological cancers. However, mutations in members of some of the other Ras subfamilies and also in genes coding for proteins in their downstream signalling cascades have been identified more recently. Most famous amongst them are mutations in *BRAF*, found frequently in melanoma and colorectal cancer amongst others. Amongst haematological cancers, mutations in *BRAF* were recently found in virtually all cases of hairy cell leukaemia and also in about 10% of cases of myeloma and less frequently in other lymphoid tumours. Also, mutations in the phosphatase *PTPN1* are present in 20–25% of Hodgkin and primary mediastinal B-cell lymphomas; whilst mutations in *RHOA* were recently identified in 25–30% of peripheral T-cell lymphomas including 75% of angioimmunoblastic lymphoma.

The P53 network

TP53 encodes the P53 transcription factor that is activated in response to different types of cellular stress and best characterised in its response to DNA damage. P53 activates a number of transcriptional networks that result in cell cycle arrest and/or activation of apoptosis. This serves to block the transmission of damaged/mutated DNA to daughter cells, preventing the accumulation of mutations that ultimately could lead to cancer. The important tumour suppressor role of P53 is evident in P53-deficient transgenic mice, which display a significantly increased frequency of cancers leading to shortened survival. In humans, *TP53* mutations disrupting one of the two P53 alleles cause the Li Fraumeni syndrome, where afflicted individuals develop cancers at an early age. Somatically acquired mutations in the *TP53* gene are found in more than 50% of human solid organ cancers. *TP53* mutation frequency is lower in haematological malignancies, found in 8–10% of AML and 4–8% of CLL. The majority of *TP53* mutations are missense changes that destroy the DNA binding ability of the P53 protein. *TP53* mutations confer poor prognosis as P53-deficient leukaemic cells can withstand genotoxic chemotherapy through failure to activate the appropriate DNA damage response that triggers apoptosis. Furthermore, the surviving P53 deficient cells have increased genomic instability that facilitates clonal evolution through acquisition of further chemotherapy-resistant mutations. Indeed, pre-leukaemic/leukaemic clones carrying *TP53* mutation can be preferentially selected following chemotherapy due to their resistance to treatment. This is evidenced by higher frequency of *TP53* mutations in leukaemias arising in patients with prior exposure to genotoxic chemotherapy. For example, up to 30% of all cases of therapy-related AML (t-AML) harbour *TP53* mutated.

The P53 protein is regulated by a number of post-translational protein modifications such as phosphorylation and ubiquitination. One such enzyme that regulates the activity of P53 is PPM1D (also known as Wip-1), a phosphatase that dephosphorylates and deactivates P53. PPM1D is itself a transcriptional target of P53, enabling a negative feedback loop upon P53 activation. The frequency of mutations in *PPM1D* follows the pattern observed for *TP53* in leukaemia, with less than 1% in de novo AML and 1–2% in MPN, and 5–10% in t-AML. Altogether, this suggests the loss of P53 pathway in leukaemia plays a greater role in providing resistance to chemotherapy than the initiation of leukaemogenesis.

Splicing factors

The initial transcription of genes produces a pre-mRNA transcript that contains both exons and introns. In order to permit protein translation from the exons, a fundamental process called splicing removes the non-coding introns and joins the remaining exons to produce a mature mRNA. In addition, splicing can also include or exclude specific exons from the mature mRNA to produce proteins with different amino acid sequences. This is known as alternative splicing and serves to expand the diversity of our genome by enabling a single gene to encode multiple protein variants. RNA splicing is coordinated in the nucleus by the

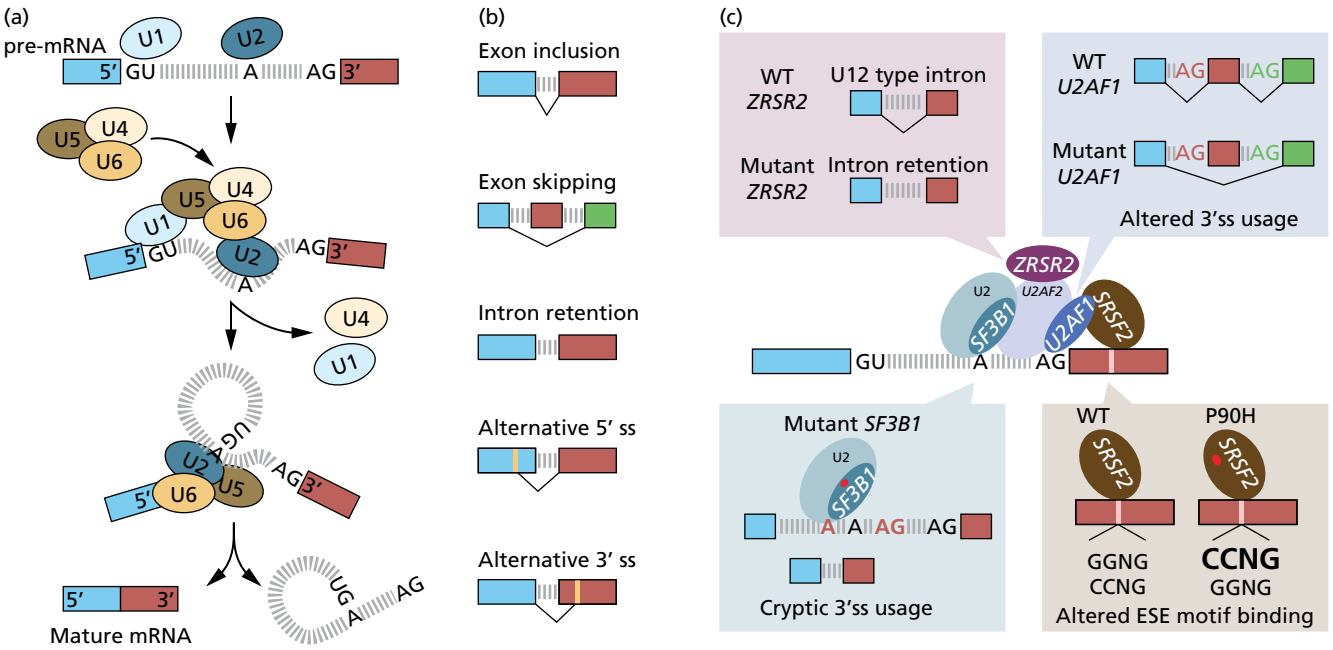

Figure 9.10 Abnormal splicing in haematological malignancies. (a) In normal splicing, the GU dinucleotides at the 5′ donor splice site (ss) of the intron are bound by the U1 small nuclear ribonucleoprotein complex (snRNP), and the A nucleotide at the splice branch site is bound by the U2 snRNP. The U4, U5 and U6 snRNP trimer is recruited and promotes cleavage at the 5′ GU donor site, followed by ligation of the GU to the A branch site to form a lariat structure. Subsequently, the RNA is cleaved at the AG dinucleotides 3′ receptor splice site to release the lariat structure, and the two flanking exons are ligated together. (b) In addition to removal of introns, alternative splicing of the pre-mRNA transcript can lead to skipping of adjacent exons, retaining introns, using alternative 5′ splice donor site (5′ ss) or 3′ splice receptor site. This leads to a diversification in the protein sequences arising from a single gene locus. (c) Mutations in the genes for splicing factors *SF3B1*, *U2AF1*, *SRSF2* and *ZRSR2* and their pathological consequence on splicing.

spliceosome, a complex of small ribonucleoprotein enzymes that catalyse the removal of introns and joining of exons (Figure 9.10).

Aberrant splicing in cancers can originate from somatic mutations in the actual sequences recognised by the splicing reaction. Paired analysis of DNA and RNA sequencing from the TGCA has shown around 1.6% of over 600,000 somatic mutations from 12 cancer types affected these splice sites. Whilst splice site mutations typically only affect the splicing of a single gene, blood cancers are enriched for somatic mutations affecting the core components of the spliceosome complex and resulting in global splicing aberration. The splicing factor genes most often mutated are *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2* (Figure 9.10c). Collectively, these mutations are most frequently identified in MDS (>65%), secondary AML progressed from MDS (>40%), CLL (10–20%) and de novo AML (5–10%). The clinical impact of spliceosome mutations varies depending on the haematological malignancy. For example, CLL harbouring SF3B1 mutations is generally associated with worse overall survival. In MDS, *SF3B1* mutations confer a more favourable prognosis with improved overall survival, whilst *SRSF2* and *U2AF1* mutations are associated with poorer prognosis. Spliceosome mutations have also been identified as drivers of clonal haematopoiesis, particularly in people aged 70 years or older. This suggests the spliceosome mutations drive clonal expansion in the context of ageing, but the mechanism by which altered splicing improves the fitness/survival of the haematopoietic clone is unknown (see clonal haematopoiesis Chapter 38).

Splicing gene mutations are heterozygous and alter the splicing activity of the affected spliceosome proteins. *SF3B1* mutations, such as K700E and K666N, result in variant SF3B1 proteins that select an upstream cryptic 3′ splice site leading to aberrant inclusion of an additional 10–15 nucleotides into the spliced mRNA transcript. The canonical function of SRSF2 is to recognise and bind the exonic splicing enhancer sequences in the RNA transcript to facilitate splicing. The vast majority of *SRSF2* mutations are non-synonymous substitutions affecting the P95 codon. These mutations produce an SRSF2 variant that alters the affinity to the exonic splicing enhancer sequence to favour the CCNG over the GGNG sequence, leading to changes recurrent splicing changes. *U2AF1* mutations also affect the recognition of the 3′ splice site, leading to usage of alternative 3′ splice sites and in many

cases to skipping of entire exons. Unlike the other three commonly mutated splice factor genes, *ZRSR2* is affected by loss-of-function mutations that are associated with increased intron retention events. The effects of altered splicing on a target gene can vary from gene expression, a shift in the physiological ratio of splice variants and/or the production of a protein with altered activity due to retention or skipping of specific exons.

The identification of the aberrantly spliced target genes that contribute to leukaemogenesis has been challenging and elusive. Spliceosome mutations can affect splicing of hundreds of different genes and it is not clear which of these, are responsible for the associated phenotypic changes. In addition, dissection of the molecular mechanisms of spliceosome mutations in murine models is complicated by the murine spliceosome factors targeting a different set of genes compared to their human counterparts. Nevertheless, the basis of some of the changes associated with splicing gene mutations is better understood. The best example of this is the formation of ring sideroblasts in *SF3B1*-mutant MDS, which is driven by altered slicing of genes involved in haem biosynthesis, namely *ABCB7*, *TMEM14C* and *PPOX*. The failure to produce adequate amounts of haem molecules leads to an excess of free iron in mitochondria, which is morphologically detectable as ring sideroblasts.

Multiple myeloma

Unlike any other somatic cell type, B and T lymphocytes undergo physiological DNA rearrangements during their development. These events are confined to the loci encoding their antigen receptor components and are required to generate the necessary antigen receptor diversity. In the case of B lymphocytes, the antigen receptor specificity is first generated through rearrangement of the V(D)J region mediated by the RAG1 and RAG2 enzymes when the developing B-cell is still in the bone marrow. In the mature circulating B-cell, the activation-induced (DNA-cytosine) deaminase (AID) protein then mediates two further processes, class-switch recombination and somatic hypermutation in the germinal centre of secondary lymphoid organs. While the former is required to change the class of the antibody produced by the B-cell (e.g. IgM to IgG), the latter introduces random point mutations in the antigen-binding site of the immunoglobulin in an attempt to increase its specificity and affinity to the target antigen. V(D)J rearrangements and class-switch recombination are achieved through generation of DNA double-stranded breaks (DSB) at specific consensus sites, deletions of part of the locus, and subsequent non-homologous end joining of the two DNA ends.

Multiple myeloma is a neoplasm of antibody-producing post-germinal centre B-lymphocytes, i.e. plasma cells. As an unwanted side-effect of their development, B-cells can occasionally acquire translocations caused by illegitimate joining of a DSB in the Ig region with a second DSB elsewhere in the genome, both caused by the activity of RAG or AID. In fact, about 40% of multiple myeloma cases show deregulated expression of oncogenes caused by their translocation next to highly active immunoglobulin (Ig) regulatory elements. This is a common theme in lymphoid malignancies in general and underscores the tight link between normal developmental processes and cancer. Notably, Ig translocations in multiple myeloma do not usually result in fusion proteins, but rather in deregulated over-expression of a full-length proto-oncogene under the control of highly active Ig enhancer elements. Deregulation of the G1/S transition is a key early molecular abnormality in myeloma, and frequently a D-group cyclin is deregulated in Ig translocations. This can be either through a direct mechanism, where *CCND1* and *CCND3* expression is driven by Ig enhancers in the t(11;14) and t(6;14), respectively, or it can occur indirectly, such as in t(14;16) where over-expression of *MAF* leads to activation of *CCND2*. The t(4;14), present in 15–20% of multiple myeloma cases, is by contrast a rare example of a translocation resulting in the simultaneous deregulation of two oncogenes, *FGFR3* on der(14) and *MMSET* on der(4). Over-expression of FGFR3 protein occurs in only approximately 70% of patients with the translocation, while *MMSET* is over-expressed in all cases. The *MMSET* gene product is a histone methyltransferase with affinity for H3K36 and H4K240, and its deregulated expression can influence DNA damage repair processes and the overall transcriptional profile of the cell. FGFR3 is a transmembrane tyrosine kinase receptor that is involved in key embryonic and adult processes including regulation of proto-oncogenic pathways such as the mitogen-activated protein kinase (RAS-RAF-MAPK), phosphatidylinositol 3-kinase (PI3K-AKT-mTOR), phospholipase Cg (PLCg), protein kinase C (PKC) and signal transducer and activator of transcription (STAT). Interestingly, activating mutations of FGFR3 and MMSET are also found in multiple myeloma and other malignancies, indicating that they may be more broadly implicated in oncogenesis.

Multiple myeloma shows multistep evolution and is almost invariably preceded by the premalignant condition, monoclonal gammopathy of unknown significance (MGUS). Chromosomal aberrations are regularly present in MGUS, indicating that Ig translocations are early events in the pathogenesis of multiple myeloma, whilst transition towards full-blown myeloma is achieved through the stepwise acquisition of additional mutations. Amongst those are deletions of chromosomes 1p, 13 and 17p, and amplification of chromosomes. Furthermore, mutations of genes involved in the MAPK signalling pathway, including *KRAS*, *NRAS*, *NF1* and the downstream protein BRAF are present in about 50% of cases, and their frequency increases in more advanced cases, again underscoring the broad relevance of this pathway to carcinogenesis.

Clonal evolution and subclonal architecture of haematological cancers

Clonal evolution of cancer

Cancer develops through the serial acquisition of somatic mutations in an individual cell and its progeny. The mutations are usually acquired in a stepwise manner, such that each time a new driver mutation is acquired, this can be seen as a cloning event. In other words, the new mutation en route to neoplasia can be seen as an event that extricates that cell from its peers, such that the onward path towards cancer continues in this individual cell's progeny. The next step in cancer evolution occurs when a new driver mutation arises in this progeny and a new clone is formed and so on. The process continues until a cell acquires a complement of driver mutations for frank malignancy to become manifest (*linear evolution*, Figure 9.11). In reality, the length of time required for this process is long enough for different individual cells within each of the serially appearing clones to acquire different mutations and continue along their own independent paths towards cancer (*branching evolution*), although one clone will usually get there first and represent the bulk of the disease. This process has been very well worked out in AML, ALL and multiple myeloma, where it is clear that although many cases follow a predominantly linear evolution, others follow a branching pattern. Of note, sometimes a very similar or even the same mutation can be acquired independently by two different subclones. This reflects a 'craving' for that mutation by the evolving cancer and the process is often referred to as *convergent evolution*. The subclonal structure of the tumour and the relationship between subclones is also relevant with regard to disease relapse and is discussed below.

Another important conundrum in cancer evolution is the order of acquisition of specific mutations. A number of studies have demonstrated such an order and AML, ALL and myeloma are again amongst the best-studied paradigms for this phenomenon. For example, in myeloma there is good evidence that chromosomal translocations and aneuploidy are usually acquired first, whilst mutations in proliferative pathways are acquired later. Also in AML, mutations affecting genes involved in DNA methylation such as *DNMT3A* and *TET2* are acquired first, whilst mutations in *NPM1* are acquired second and proliferative mutations are again acquired late.

There are two theories proposed for this recurrent order of acquisition: the *constraint theory* argues that a certain mutation has to happen first in order to 're-wire' the cell so that the second can give additional growth advantage, which would not be present in the absence of the first mutation. By contrast, the *opportunity theory* argues that the first mutation imbues features such as increased proliferation or accelerated level of mutagenesis,

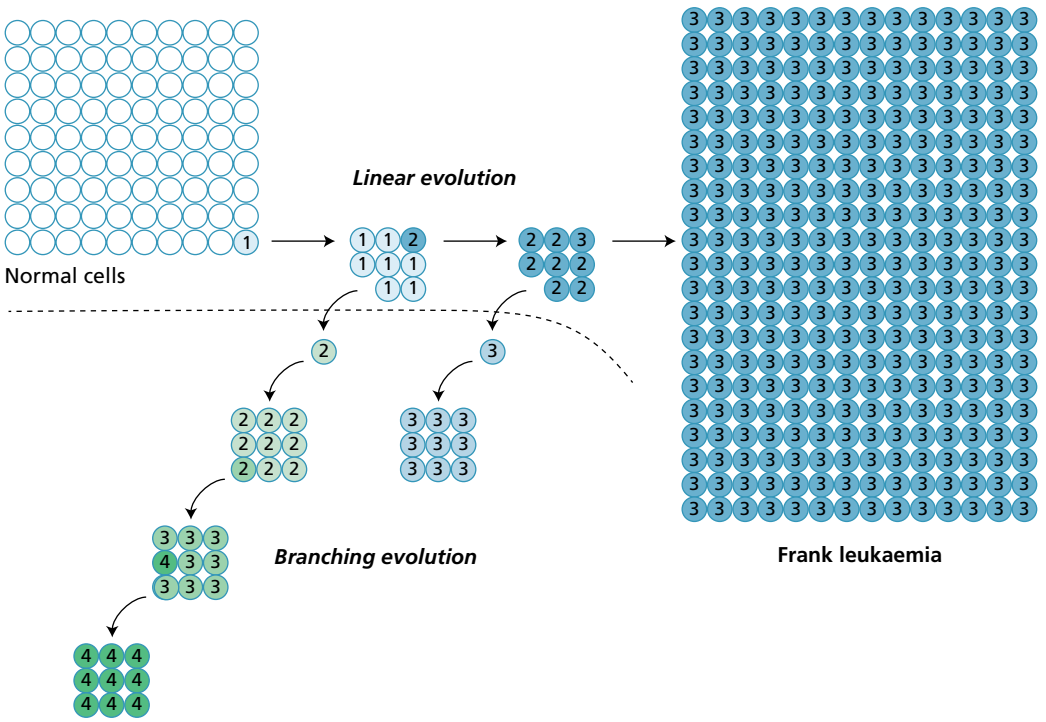

Figure 9.11 Linear and branching evolution.

that confer greater opportunity for the second mutation to be acquired. Additionally, in the latter theory, the order can be reversed, but this will happen a lot less often as there will be less opportunity. It is not yet clear which of the two theories holds true, or if indeed they both do, depending on the type of mutation; however, there is some evidence directly supporting the second. Namely, *DNMT3A* is usually acquired before NPM1c in AML; however, occasional cases of AML may acquire the *NPM1* first and *DNMT3A* mutation second. Another interesting observation about mutations that are usually acquired first in clonal evolution is the fact that they are found frequently in related, but distinct, neoplasms. For example, *TET2* mutations are seen in AML, MDS, PV, ET, CMML, systemic mastocytosis (SM), chronic neutrophilic leukaemia (CNL) and even in B- and T-cell lymphomas. This suggests that these mutations provide a fertile ground for 'second' mutations to be acquired that will eventually dictate the resulting phenotype of the cell. In fact recent studies have revealed that mutations in particular genes, most commonly *DNMT3A*, *TET2*, *ASXL1* and *JAK2*, can establish a detectable clone of haemopoietic cells in healthy individuals lacking any evidence of haematological cancer (see Chapter 38 for more details). This phenomenon of 'clonal haemopoiesis', becomes more frequent with age and can be detected in the majority of people aged 90 years or older. Cells with such clone-founder mutations can then acquire additional mutations, such as those affecting *NPM1* and *FLT3* in the case of mutant *DNMT3A*-driven clones, that can generate an acute leukaemic expansion. Treatment of the acute leukaemia may then result in remission, but original clone harbouring *DNMT3A* mutations only can persist untouched by chemotherapy and may give rise to late disease relapses.

Impact of clonal structure on treatment and relapse

The parallels between the clonal development of cancer and Darwinian evolution by natural selection are particularly instructive when studying anticancer treatment. Having escaped physiological controls, cancer cells effectively take charge of their own destiny, not unlike a species of individuals doing so in nature. The administration of chemotherapy can then be seen as a serious menace that threatens the 'species' with extinction. Species evade such extinction by leveraging their heterogeneity; in other words, they rely on some individuals resisting the onslaught and surviving to regenerate the species. The simultaneous presentation of multiple different hazards to a species increases the likelihood of extinction, as witnessed by the effectiveness of combination antiretroviral therapy against HIV and combination chemotherapy regimens against paediatric ALL.

Nevertheless, only a minority of adult haematological cancers are curable. Clinically, treatment failure can present itself as resistant disease, partial response or relapse, but effectively in all instances, this amounts to variable numbers of cancer cells surviving chemotherapy. Advances in genomics have demonstrated that tumours represent an admixture of competing subclones with distinct genotypes, which have different abilities to resist chemotherapies. The different types of treatment failure reflect the numbers of cells resisting therapy, with resistant disease and partial response indicating that the bulk of cells were resistant or partially resistant to treatment. Relapse after complete remission indicates that only a small number of cells survived and this talks directly to the subclonal architecture of cancers.

As first shown in AML and subsequently in multiple myeloma and CLL, sequencing of serial samples at diagnosis and relapse reveals tumour subclones that are present only in the relapsed sampled, only in the diagnostic sample or in both (Figure 9.12). Despite the fact that treatment can eradicate subclones in some cases, mutations representing the founding clone are invariably presented at relapse, often associated with subclones containing new mutations not apparent at diagnosis. This clearly shows how the same treatment can have a differential effect on tumour subclones. From this perspective, chemotherapy can be seen as an 'evolutionary bottleneck' that either allows low-frequency chemoresistant tumour cells to survive and reconstitute the bulk of disease at relapse, or promotes evolution of clones through acquisition of new mutations associated with chemoresistance.

Usually, novel mutations identified first in chemoresistant clones can be retrospectively identified in the pre-treatment sample when sensitive techniques are used, although in some instances such mutations appear to be unique to the relapsed clone. Arguably this is only of academic significance, as in both instances the disease relapses with mutations associated with chemoresistance. However, there is some evidence that relapsed clones may harbour signatures of mutational processes attributable to chemotherapy, suggesting therefore that if non-mutagenic treatment had been used, relapse may not have ensued. It is not as yet clear if this applies to some or many cases of relapsed haematological cancers.

When trying to optimise treatment against relapsed disease, one can consider that tumours relapsing after a certain latency and without any change in their subclonal structure may be sensitive to the same treatment they responded to previously, whereas samples showing early relapse in association with acquisition of new driver/resistance mutations may require different therapy. Furthermore, the identification of clinically actionable mutations may lead clinicians to favour a particular targeted treatment over another. Finally, large studies correlating the genotype of subclones with treatment response could predict which combination of drugs will be most effective, and thus allow the clinician to deliver a 'tailored' treatment for maximal efficacy to individual patients. Based on its substantial potential for guiding treatment, it is likely that the usage of NGS techniques for diagnosis and prognosis will increase significantly to assist the future management of haematological malignancies.

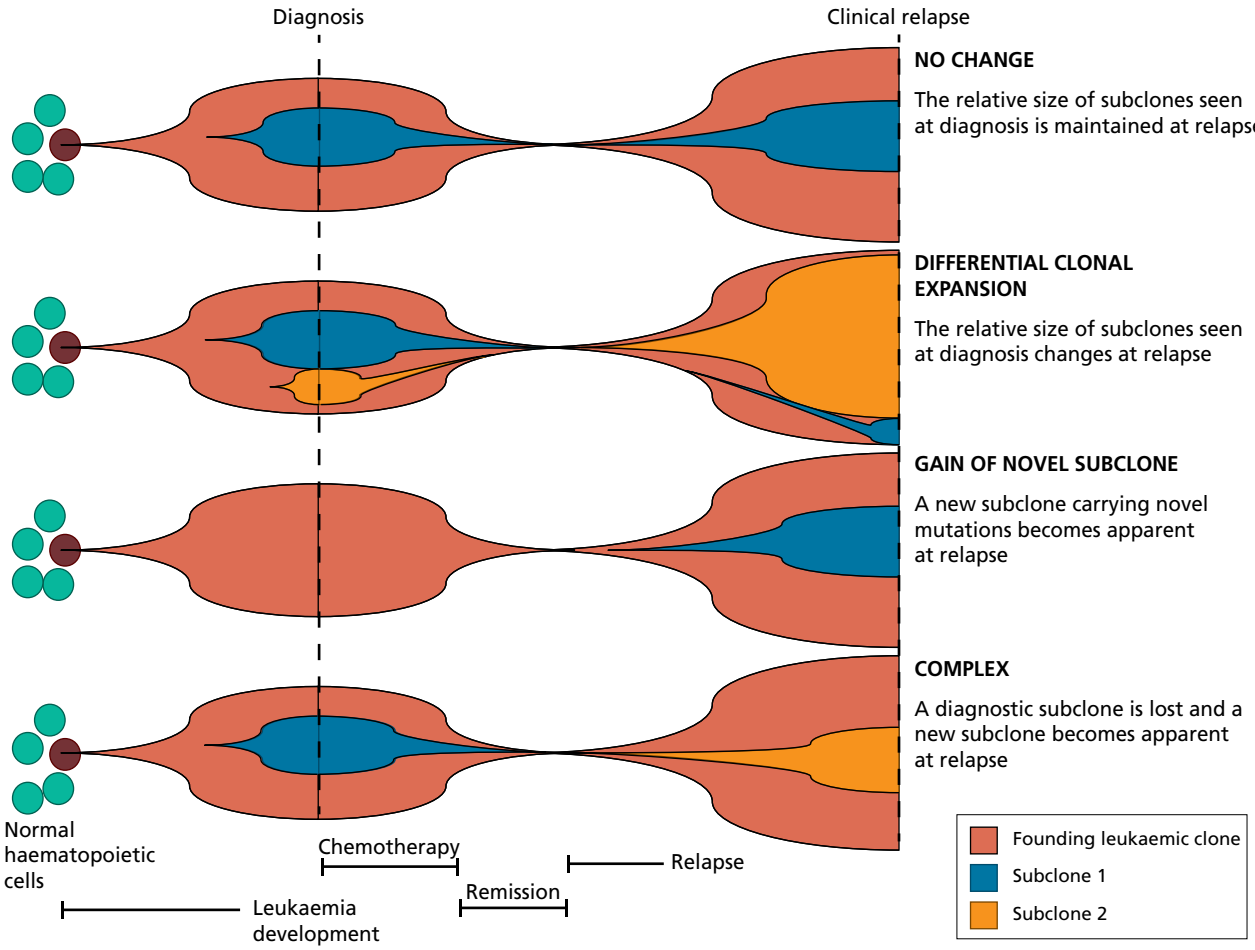

Figure 9.12 Clonal evolution of cancer subclones in relation to treatment.

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CHAPTER 10

Laboratory diagnosis of haematological neoplasms

10

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Introduction

The diagnosis of a haematological neoplasm usually starts from a clinical suspicion, although for chronic leukaemias, the diagnosis is sometimes an incidental one. A blood count and blood film are essential first steps whenever a haematological neoplasm is suspected. The next step in the diagnostic process depends on the clinical features and the specific condition that is suspected. In this chapter, we discuss the laboratory techniques that are available and how they are integrated into an efficient diagnostic pathway (Figure 10.1). It is of crucial importance that all laboratory investigations are done with an awareness of the medical history and physical findings. It is also essential that a conclusion as to diagnosis is based on integrating the results of all laboratory investigations and imaging in the context of the clinical features. Achieving this can be a challenge if investigations are done in different laboratories on different sites. One possible solution, if all procedures are not performed in a single laboratory, is that a haematologist/haematopathologist should take the lead in making a final diagnosis when the diagnosis depends primarily on the blood and bone marrow and that a haematopathologist/histopathologist should take the lead when the diagnosis depends primarily on a biopsy of another tissue. Three important factors contribute to accurate diagnosis:

1 The provision of very detailed clinical information to laboratories

2 The correct sample promptly delivered, e.g. blood or bone marrow aspirate specimen (appropriately anticoagulated), blood and bone marrow films, trephine or fine-needle biopsy specimen, should all be sent to the laboratory in the shortest possible time

3 The integration of information technology across different sites dealing with diagnostic samples from a single patient.

Consideration should also be given to the need for centralised diagnosis by a panel of experts when experience has shown that misdiagnosis is common, for example in lymphoma. It is also necessary to ensure that a diagnosis is achieved in a timely manner, particularly when treatment may be urgent, as in acute leukaemias and aggressive lymphomas.

Blood count and blood film

The most important information that must be extracted from the blood count is the white cell count, the haemoglobin concentration and the platelet count. The red cell indices should also be noted since macrocytosis and, less often, microcytosis can occur in haematological neoplasms. The automated instrument will generally provide a differential count and 'flags' indicating the likely presence of blast cells, abnormal lymphocytes or granulocyte precursors. An abnormal total or differential count or a low platelet count should always be verified on a blood film and a blood film should also be examined when there are relevant 'flags'. Even if the blood count is normal, a blood film should be examined for the presence of abnormal cells in any patient with lymphadenopathy, splenomegaly, skin infiltration or any other reason to suspect a haematological neoplasm.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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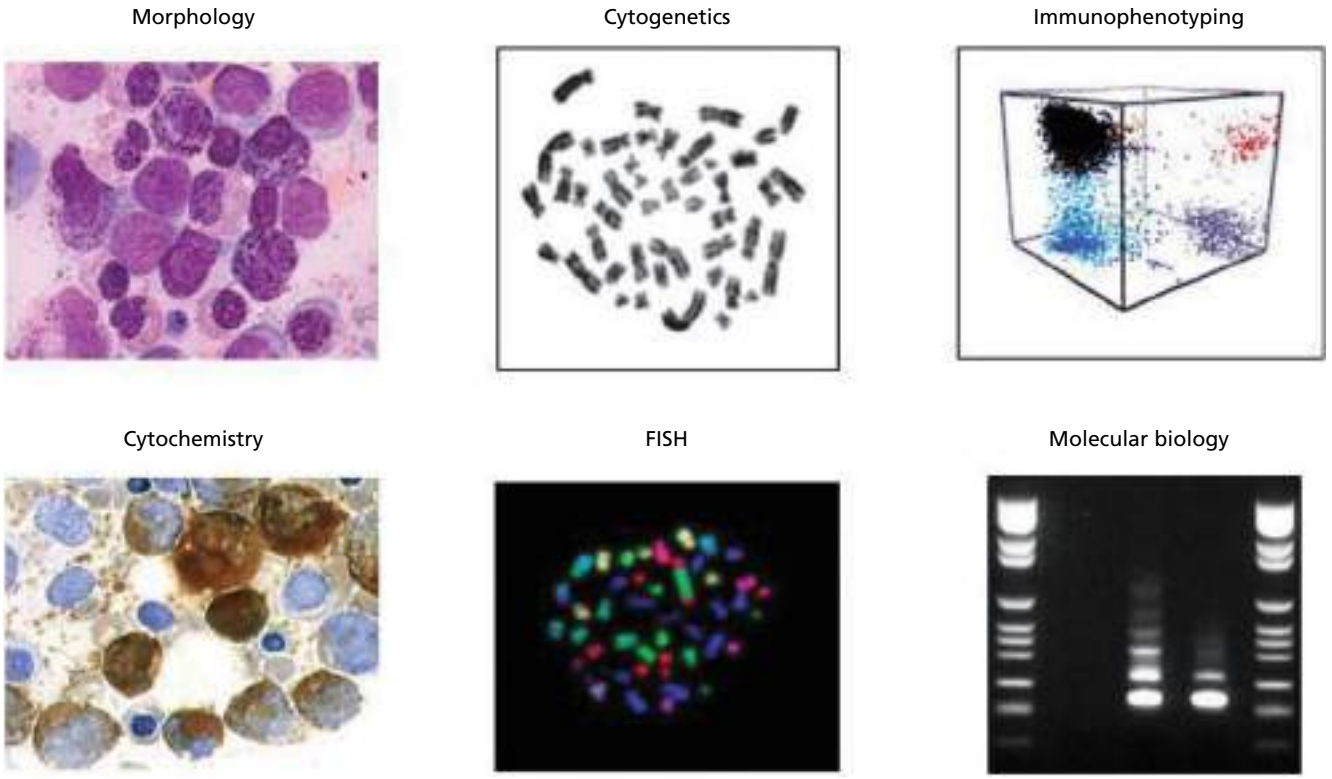


Figure 10.1 The principal laboratory methods employed in haematological diagnosis.

The blood film has two major roles. In some circumstances, it provides strong evidence of a specific diagnosis that can be confirmed on further testing. In others, it suggests a differential diagnosis and indicates the appropriate direction of further tests. A blood film also has the advantage that a rapid provisional diagnosis can be made, something that is important when treatment is urgent. For example, when there are circulating neoplastic cells a rapid provisional diagnosis of Burkitt lymphoma (Figure 10.2) and acute hypergranular promyelocytic leukaemia (Figure 10.3a) can be made. Acute microgranular/hypogranular promyelocytic leukaemia (Figures 10.3b and 10.4) should also be suspected from the blood film. Even very infrequent neoplastic cells can suggest the correct diagnosis, for example in hairy cell leukaemia. Other diagnoses that may be strongly suggested by the blood film include most subtypes of acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL) (Figure 10.5), chronic myeloid leukaemia (CML) (Figure 10.6), follicular lymphoma, splenic marginal zone lymphoma (when villous lymphocytes are present), large granular lymphocytic leukaemia, Sézary syndrome and plasma cell leukaemia.

Sometimes the blood film suggests only a differential diagnosis. Although the cytological features of some cases of acute lymphoblastic leukaemia (ALL) (Figure 10.7) are very likely to indicate that this is the diagnosis, this is not so of other cases (Figure 10.8), which can be cytologically very similar to AML with minimal differentiation or AML without maturation (Figures 10.9 and 10.10). Similarly, acute monoblastic leukaemia (Figure 10.11) can be cytologically indistinguishable from large-cell lymphoma or even plasma cell leukaemia.

In suspected myeloid malignancy, the blood film is important for the detection of myelodysplastic features, particularly

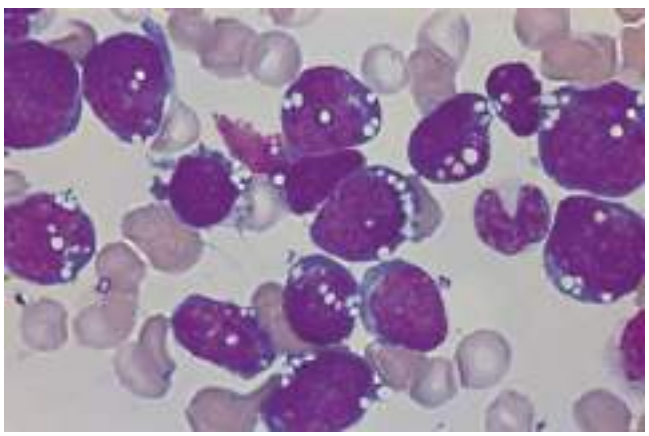
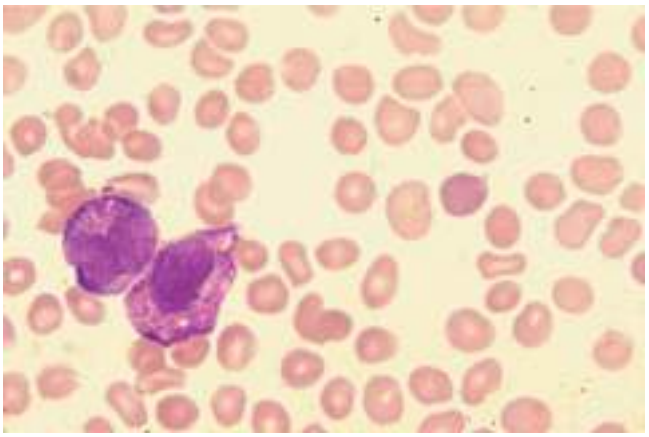


Figure 10.2 Burkitt lymphoma showing blast-like cells with strongly basophilic, heavily vacuolated cytoplasm. The cytological features were once designated 'FAB L3 ALL' but the immunophenotype is that of a mature B cell. Bone marrow film, May–Grünwald–Giemsa (MGG).



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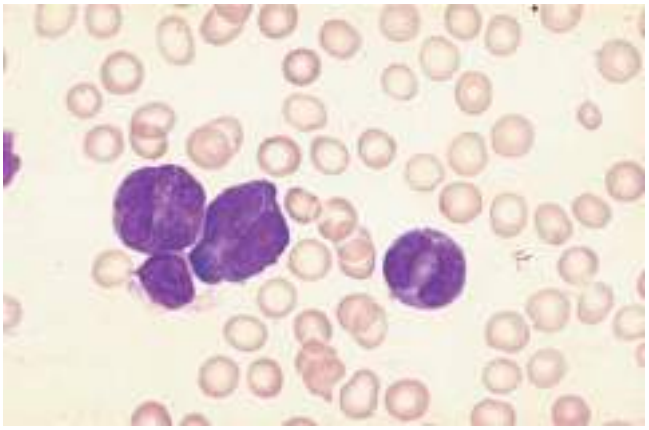


(b)

Figure 10.3 Blood films of two patients with acute promyelocytic leukaemia: (a) acute hypergranular promyelocytic leukaemia. (b) hypogranular/microgranular variant of promyelocytic leukaemia showing typical bilobed nuclei. These two subtypes of AML fall into a single category of 'AML with defining genetic abnormalities' in the 2022 WHO classification and are similarly a defined category in the International Consensus Classification (ICC). Peripheral blood films, MGG.

hypogranular and pseudo-Pelger–Huët neutrophils (Figure 10.12). Sometimes the blood film shows only non-specific, but nevertheless useful, features, for example eosinophilia, rouleaux formation, red cell agglutinates or cryoglobulin deposition.

Whatever the findings on the blood count and film, confirmatory tests are needed. The nature of these will vary according to circumstances, but several useful algorithms are guided by information that can be gained from the peripheral blood.

Bone marrow aspirate

A bone marrow aspirate is indicated in virtually all patients with suspected ALL, AML (Figure 10.13), CML, myelodysplastic neoplasm/syndrome (MDS) (Figure 10.14) or multiple myeloma (Figure 10.15). However, a bone marrow aspirate is not necessary for the diagnosis of CLL. The diagnosis of acute promyelocytic leukaemia may be more readily made on an aspirate than on the peripheral blood since, particularly in the hypergranular form, there may be few circulating leukaemic cells and in the microgranular/hypogranular form, there may be more hypergranular cells in the bone marrow, thus facilitating the diagnosis. The characteristic cytological features of AML with inv(16) or t(16;16) are apparent in the bone marrow (Figures 10.16 and 10.17), but less so in the peripheral blood, although the presence of eosinophils or eosinophil myelocytes

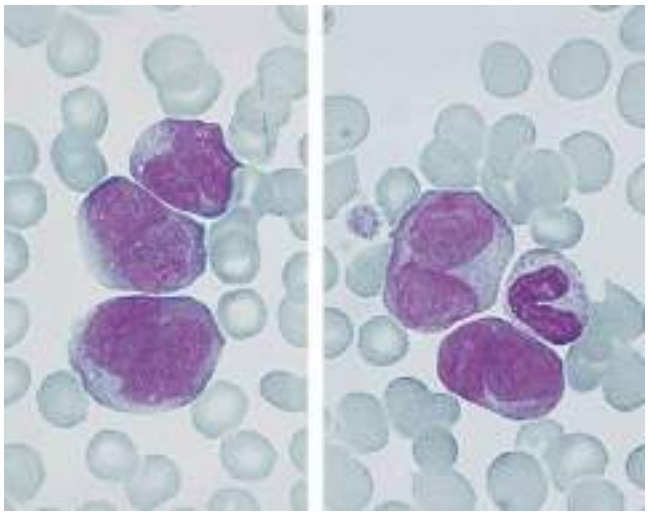


Figure 10.4 Acute hypogranular/microgranular variant of promyelocytic leukaemia showing a range of nuclear forms, but note the typical bilobed nucleus in which the two lobes are joined by an isthmus. Bone marrow film, MGG.

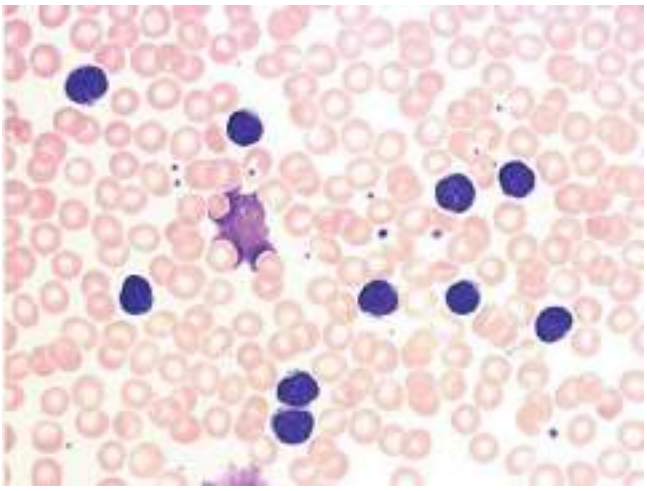


Figure 10.5 Chronic lymphocytic leukaemia showing mature small lymphocytes and a smear cell. Peripheral blood film, MGG.

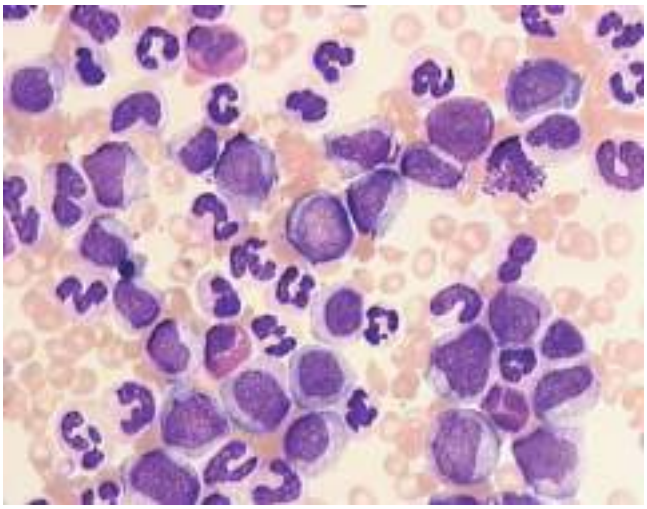


Figure 10.6 MGG-stained peripheral blood film of chronic myeloid leukaemia (*BCR::ABL1*-positive), showing the typical spectrum of granulocytic cells from immature to mature, including a basophil and some eosinophils.

with large basophilic granules is suggestive. In the acute leukaemias and in CML and other myeloproliferative neoplasms, a bone marrow aspirate provides material for cytogenetic analysis as well as for morphological assessment (Figure 10.18). Successful cytogenetic investigations from peripheral blood are only worth considering if immature leukaemic cells are present, but analysis of bone marrow cells is far more likely to be useful.

Bone marrow aspiration may also be indicated in patients with unexplained cytopenia or a leucoerythroblastic blood film. An aspirate can be useful in lymphoma diagnosis, particularly if accompanied by a trephine biopsy, and can provide suitable material for immunophenotyping and for fluorescence *in situ* hybridisation (FISH).

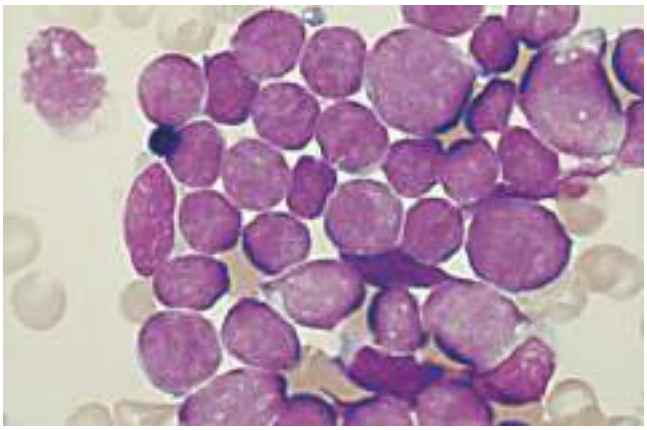


Figure 10.7 Acute lymphoblastic leukaemia showing blast cells that vary in size, but which otherwise have fairly uniform cellular characteristics; the nucleocytoplasmic ratio is high and there are small inconspicuous nucleoli. Bone marrow film, MGG.

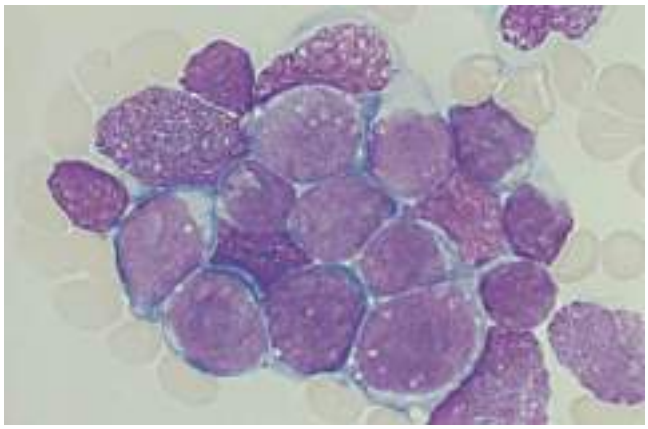


Figure 10.8 Acute lymphoblastic leukaemia showing medium-sized and large pleomorphic blast cells with one or two prominent nucleoli; there are no characteristics that identify the likely lineage. Bone marrow film, MGG.

Bone marrow trephine biopsy

A bone marrow trephine biopsy is indicated whenever there is a 'dry tap' or the aspirate obtained is aparticulate or dilute. A core biopsy is often necessary in hairy cell leukaemia, acute

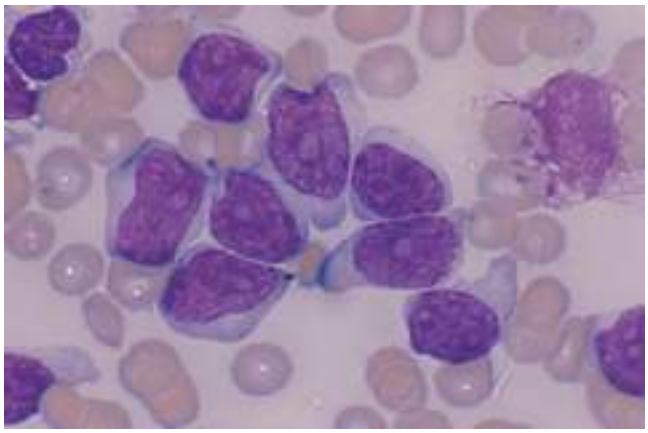


Figure 10.9 Acute myeloid leukaemia with minimal differentiation showing large blast cells with large prominent nucleoli; there are no cytological features that identify the lineage and Sudan black B and myeloperoxidase cytochemical stains were negative. On immunophenotyping, all B- and T-lineage markers were negative, but there was expression of CD13 and CD33. Without immunophenotyping such a case could not be distinguished from acute lymphoblastic leukaemia. Bone marrow film, MGG.

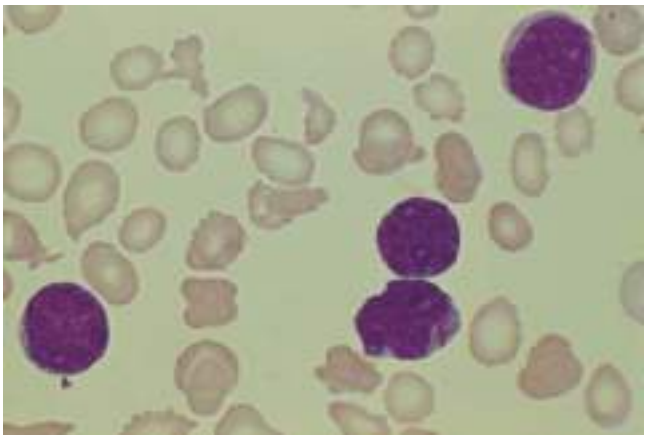


Figure 10.10 Acute myeloid leukaemia without maturation showing small- to medium-sized blast cells with a high nucleocytoplasmic ratio; one contains an Auer rod. Sudan black B and myeloperoxidase were positive. In the absence of Auer rods and granules, such cases of acute myeloid leukaemia may be indistinguishable from acute lymphoblastic leukaemia without the aid of cytochemistry or immunophenotyping. Peripheral blood film, MGG.

megakaryoblastic leukaemia (Figure 10.19) and in patients being investigated for pancytopenia, a leucoerythroblastic blood film or a suspected myeloproliferative neoplasm. If the bone marrow is being examined for suspected non-Hodgkin lymphoma (NHL), a trephine biopsy is always indicated since an aspirate may be normal cytologically and immunophenotypically despite there being focal infiltration readily detectable on a core biopsy. Hodgkin lymphoma is very rarely detected in an aspirate, whereas a trephine biopsy specimen may provide the primary diagnostic material, particularly in patients with HIV infection who can present with widespread disease. A trephine biopsy should be part of the initial investigation of suspected multiple myeloma; sometimes this confirms the diagnosis when the aspirate findings are equivocal and, even when this is not so, it is useful to have a baseline biopsy to compare with post-treatment investigations. A trephine biopsy is not generally necessary in CLL, whereas it is diagnostically important in suspected essential thrombocythaemia and polycythaemia vera and is essential in primary myelofibrosis and systemic mastocytosis. In CML, a trephine biopsy is not routinely required but may be useful in selected cases to assess bone marrow fibrosis.

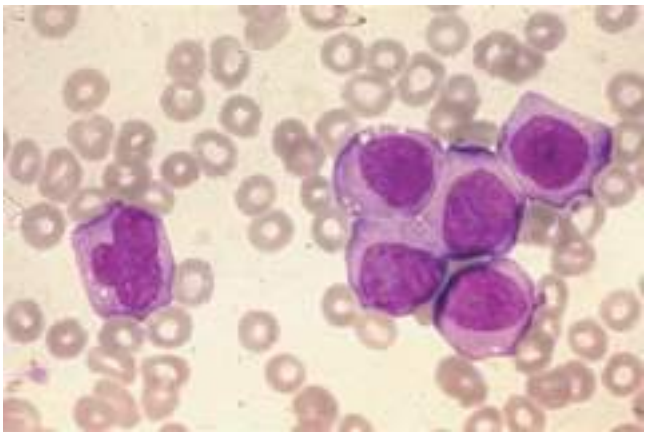


Figure 10.11 Acute monoblastic leukaemia associated with t(9;11)(p21.3;q23.3); *KMT2A::MLLT3* showing large cells with plentiful cytoplasm; one cell shows nuclear lobulation (defining it as a promonocyte in the WHO classification). In the 2022 WHO classification, this type of acute myeloid leukaemia is a specific entity within the group 'AML with defining genetic abnormalities' and similarly it is a genetic category in the ICC. Bone marrow film, MGG.

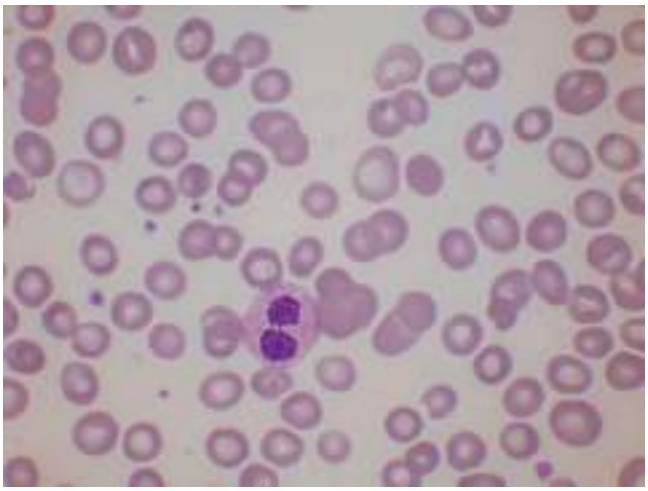


Figure 10.12 A pseudo-Pelger–Huët anomaly in a neutrophil of a patient with a myelodysplastic syndrome. Peripheral blood film, MGG.

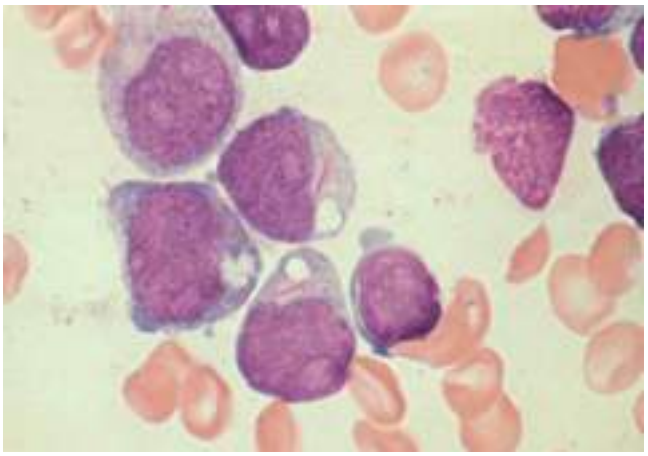


Figure 10.13 Acute myeloid leukaemia showing leukaemic blast cells, several with Auer rods. Bone marrow film, MGG.

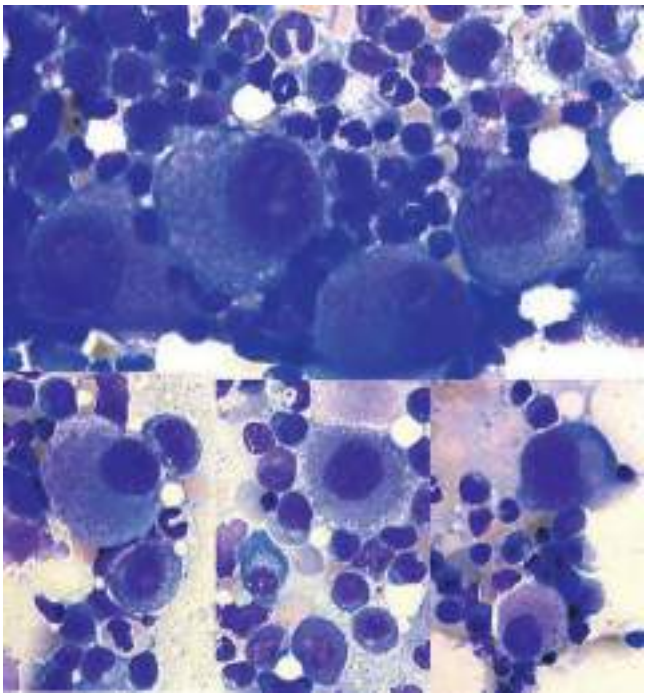


Figure 10.14 Composite image of myelodysplastic syndrome showing the hypolobulated megakaryocytes characteristic of the 5q– syndrome; myelodysplastic neoplasm with low blasts and isolated del(5q) is a specific category in the 2022 WHO classification, which in the ICC is designated MDS with del(5q). Bone marrow film, MGG.

There are other indications for trephine biopsy that are more controversial. This procedure may be necessary in ALL or AML if no aspirate or a poor aspirate is obtained. However, if there are numerous circulating neoplastic cells and if a cellular aspirate is obtained, it does not yield any important extra information. A trephine biopsy should be avoided in cases of suspected acute promyelocytic leukaemia due to the risk of bleeding. In suspected MDS, useful extra information that may contribute to the diagnosis is more likely since marrow architecture is often abnormal and immature ($CD34^+$) cells may be detected only on histology or immunohistochemistry.

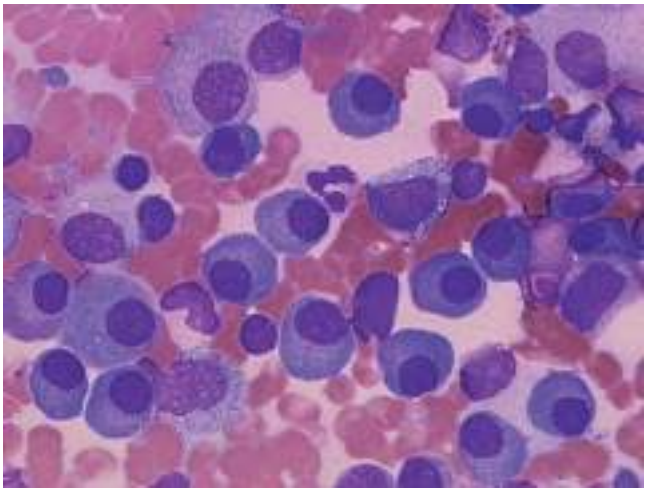


Figure 10.15 Multiple myeloma (plasma cell myeloma) showing virtual effacement of the marrow by myeloma cells. Bone marrow film, MGG.

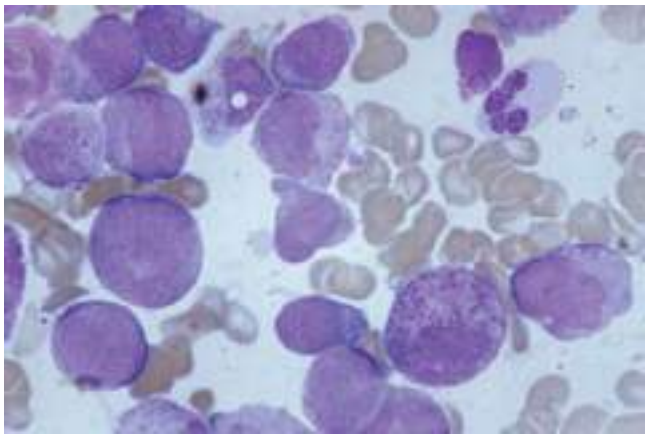


Figure 10.16 Acute myeloid leukaemia (AML) associated with inv(16)(p13.1q22); *CBFB::MYH11* showing myelomonocytic leukaemia with an abnormal eosinophil precursor that has proeosinophilic (purple-staining) granules. In the 2022 WHO classification this subtype of AML, together with similar cases with t(16;16), constitutes a specific entity within the category 'AML with defining genetic abnormalities' and is similarly an ICC category. Bone marrow film, MGG.

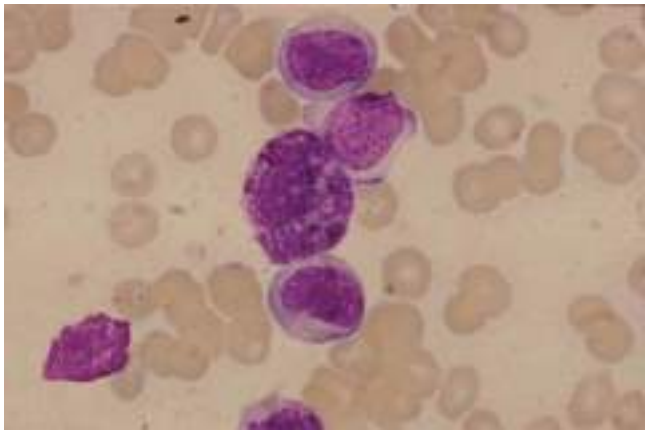


Figure 10.17 Acute myeloid leukaemia (AML) associated with t(16;16)(p13.1;q22); *CBFB::MYH11* showing blast cells and an abnormal eosinophil precursor with pro-eosinophilic granules. In the 2022 WHO classification this subtype of AML, together with similar cases with inv(16), constitutes a specific entity within the category 'AML with defining genetic abnormalities' and is similarly an ICC category. Bone marrow film, MGG.

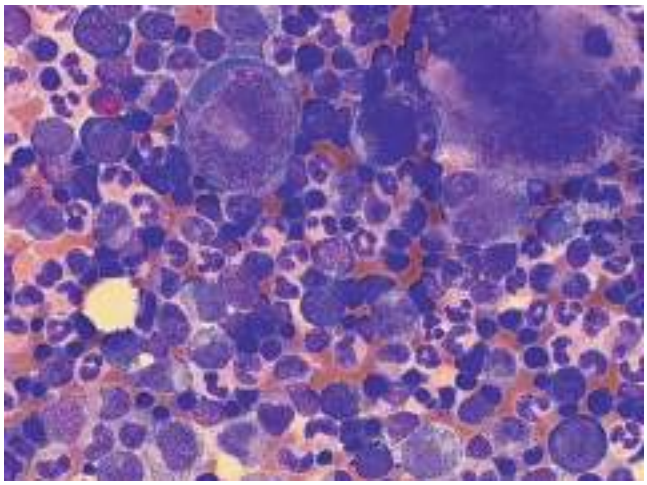


Figure 10.18 Polycythaemia vera with a *JAK2* V617F mutation showing panmyelosis; the large hyperlobulated megakaryocyte (top right) shows emperipolesis. Bone marrow film, MGG.

Cytochemistry

With advances in immunophenotyping and other techniques, the role of cytochemistry in haematological diagnosis has declined considerably. A limited role remains. In acute leukaemia, cytochemistry is indicated if there is limited access to immunophenotyping or if delay in obtaining results is expected.

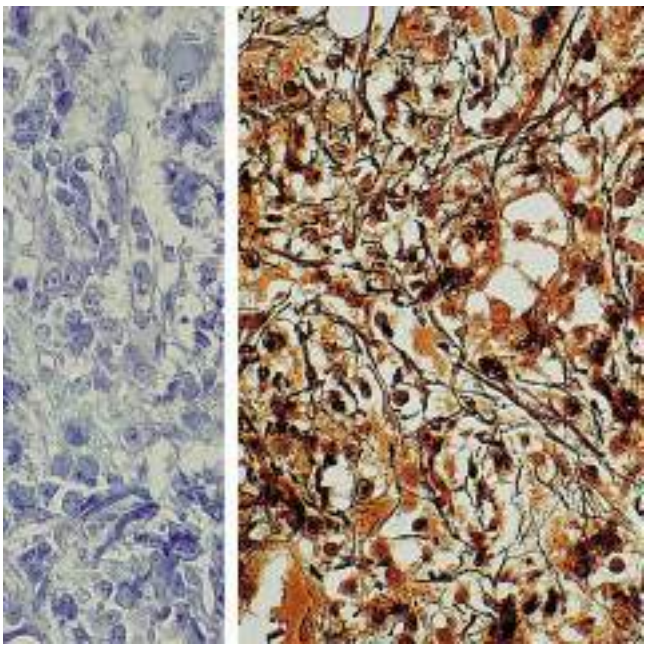


Figure 10.19 Acute megakaryoblastic leukaemia (FAB M7 subtype). Bone marrow trephine biopsy shows blast cells (left) and increased reticulin deposition (right).

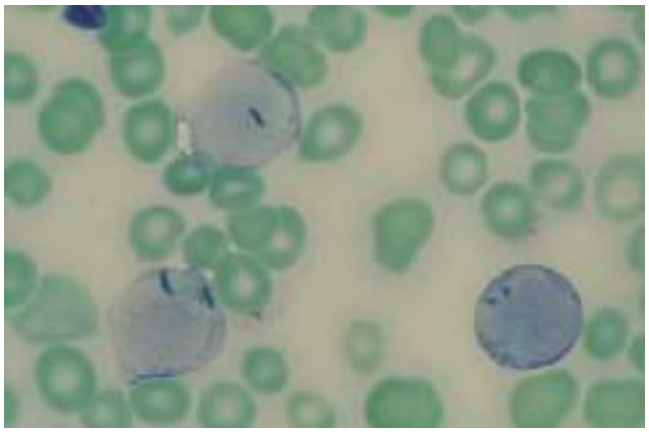
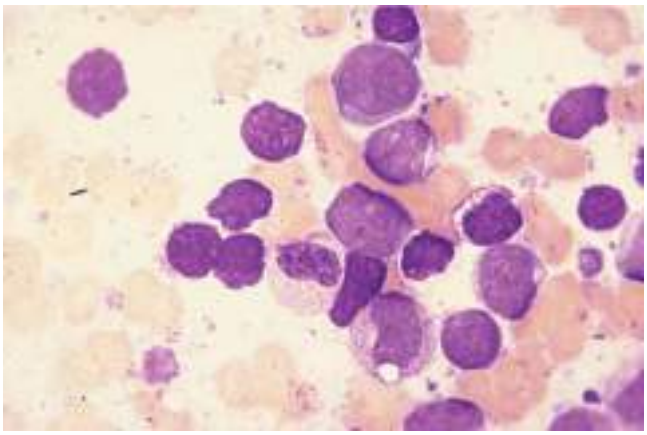


Figure 10.20 Acute myeloid leukaemia (AML) without maturation showing Auer rods. This patient had the translocation t(8;21) (q22;q22.1); *RUNX1::RUNX1T1*, identifying a specific entity within the 2022 WHO category 'AML with defining genetic abnormalities'; it is similarly a specific category in the ICC Peripheral blood film, myeloperoxidase reaction.

For example, the 2022 WHO category of AML without maturation can be distinguished from ALL by means of a Sudan black B or myeloperoxidase stain (Figure 10.20) and acute monoblastic/monocytic leukaemia can be distinguished from large-cell lymphoma by means of a non-specific esterase stain (Figure 10.21). Careful attention to cytological detail usually permits the distinction between the hypogranular/microgranular variant of acute promyelocytic leukaemia and acute monocytic leukaemia, but if there is any doubt the former diagnosis is supported by strong reactions for Sudan black B, myeloperoxidase and chloroacetate esterase and the latter by a positive non-specific esterase reaction. Rarely, a specific diagnosis of a subtype of AML is aided by demonstration of blast cells of basophil lineage

Figure 10.21 Acute monoblastic leukaemia showing strong reaction for non-specific esterase, which confirms the monocytic differentiation. Cytospin preparation, α-naphthyl-acetate esterase.



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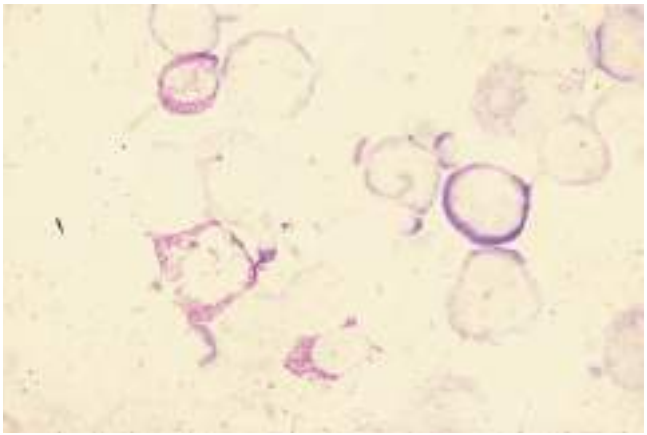

(b)

Figure 10.22 Acute myeloid leukaemia (AML) associated with t(6;9)(p23;q34.1); *DEK::NUP214,* identifying a specific entity within the 2022 WHO category 'AML with defining genetic abnormalities', which is also a defined ICC category: (a) MGG-stained bone marrow film shows blast cells and two degranulated basophil precursors. (b) Toluidine blue-stained bone marrow film demonstrates metachromasia.

showing metachromatic staining with a toluidine blue stain (Figure 10.22). Perls stain for iron remains a very important cytochemical stain in suspected MDS (Figure 10.23).

Periodic acid–Schiff and acid phosphatase reactions can help in the diagnosis of ALL (Figures 10.24 and 10.25), but reactions are not specific and we do not recommend their continued use in a routine diagnostic setting if immunophenotyping is available. A neutrophil alkaline phosphatase score is likewise redundant in the diagnosis of CML, which now depends on cytogenetic and molecular genetic analysis. A tartrate-resistant acid phosphatase reaction can confirm a diagnosis of hairy cell leukaemia, but is no longer necessary, as long as the immunophenotyping laboratory has an appropriate range of antibodies for this purpose; immunohistochemistry can be performed in the case of a 'dry tap' or *BRAF* mutation analysis can be performed.

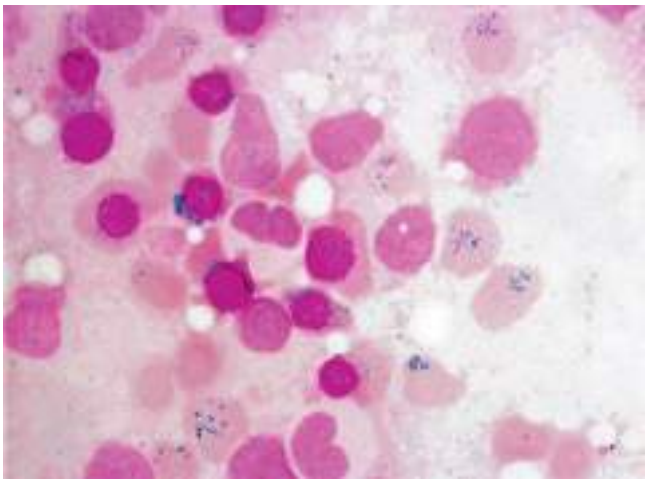

Figure 10.23 Ring sideroblasts and Pappenheimer bodies in erythrocytes in a myelodysplastic syndrome. Bone marrow aspirate, Perls stain.

Histology

Microscopic examination of sections of tissues other than bone marrow remains an essential technique for the diagnosis of many lymphomas. It may also be necessary for the diagnosis of myeloid sarcoma. Immunohistochemistry (see below) is an essential part of histological assessment for haematological neoplasms.

Flow cytometric immunophenotyping

Immunophenotyping can be based on flow cytometry, immunohistochemistry (on tissue sections) or immunocytochemistry (on cytospin preparations or films). The first two of these

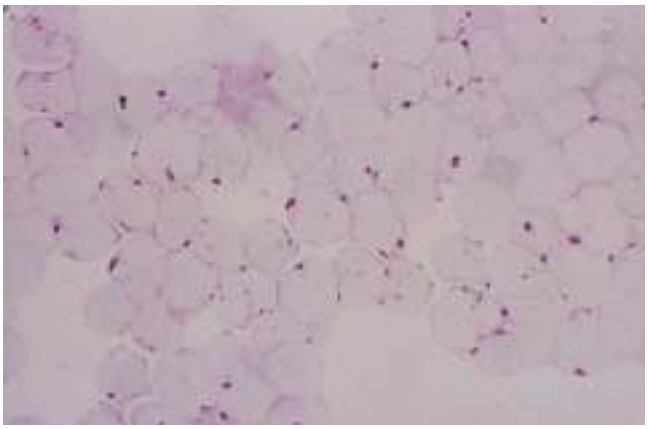

Figure 10.24 Acute lymphoblastic leukaemia showing block positivity to periodic acid–Schiff (PAS) stain. Cytospin preparation of cerebrospinal fluid, PAS stain.

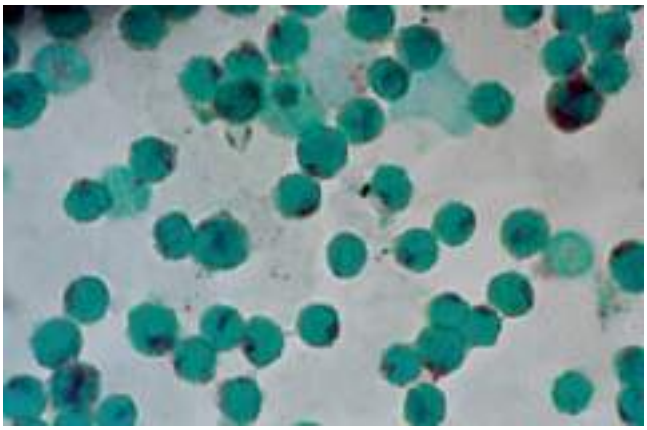

Figure 10.25 T-lineage acute lymphoblastic leukaemia showing focal (Golgi zone) positivity for acid phosphatase in lymphoblasts. Cytospin preparation, acid phosphatase reaction.

techniques are of major importance in haematological diagnosis. Flow cytometry is now carried out using multichannel instruments that permit the simultaneous assessment of forward light scatter (indicative of cell size), sideways light scatter (indicative of granularity and cell complexity) and the expression of 10 or more surface membrane antigens, all simultaneously in one vial (Figure 10.26). With the addition of techniques to 'permeabilise' the cells, cytoplasmic and nuclear antigens can also be studied. When there are numerous circulating neoplastic cells, immunophenotyping is conveniently done on a peripheral blood sample. Otherwise, it can be done on a bone marrow aspirate or on a cell suspension from any infiltrated tissue. The main indications for immunophenotyping are as follows:

1 Confirmation of a diagnosis of ALL and its subclassification (including recognition of early T-cell precursor acute lymphoblastic leukaemia) and identification of an aberrant immunophenotype (leukaemia-associated immunophenotype) that can be used to monitor measurable residual disease (MRD).

2 Confirmation of a diagnosis of AML with minimal differentiation and acute megakaryoblastic leukaemia and, if not already confirmed by cytochemistry, confirmation of a diagnosis of acute monoblastic leukaemia; support for a diagnosis of acute promyelocytic leukaemia by demonstration of lack of expression of CD34 and especially HLA-DR and by non-specific background autofluorescence observed in the isotype controls; identification of leukaemia-associated immunophenotype that can be used to monitor MRD.

3 Identification of mixed phenotype acute leukaemia.

4 Demonstration of light chain restriction, providing presumptive evidence of monoclonality, in B-lineage disorders.

5 Confirmation of a diagnosis of CLL and monitoring of MRD.

6 Confirmation of a diagnosis of NHL rather than CLL, and support for specific diagnoses such as follicular lymphoma (CD10 often positive) or mantle cell lymphoma (CD5 and nuclear cyclin D1 positive) (see also Chapter 23).

7 Confirmation of a diagnosis of hairy cell leukaemia by demonstration of expression of all or most of CD11c, CD25, CD103 and CD123; CD200 is also expressed (see also Chapter 23).

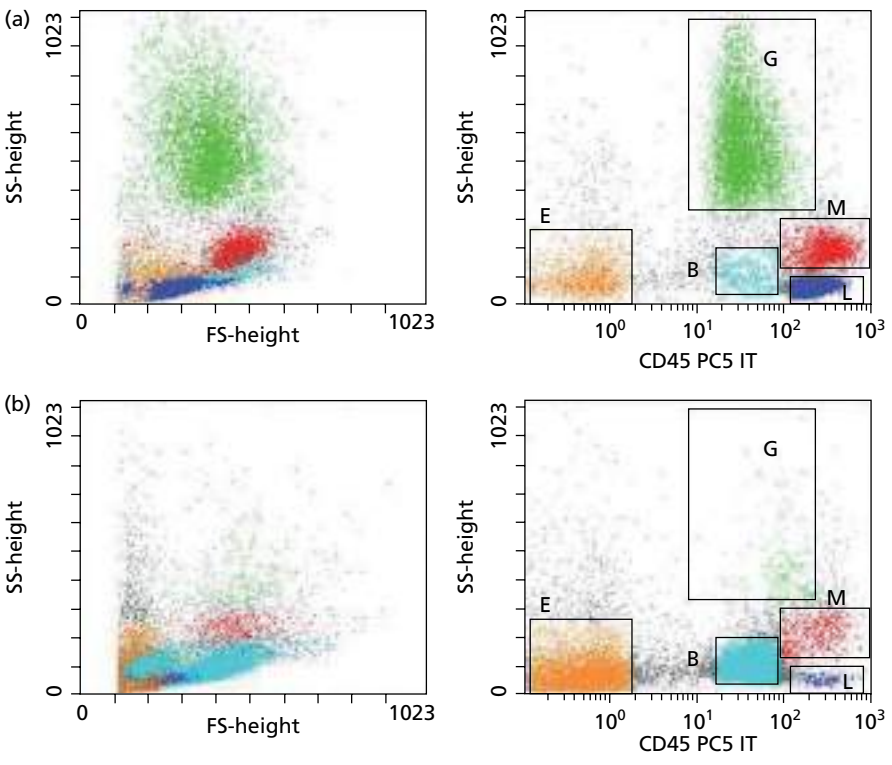

Figure 10.26 Flow cytometry immunophenotyping showing improvement of separation of populations by CD45 and sideways light scatter (SSC) gating. Forward light scatter (FSC) is also shown. (a) Normal bone marrow (left, SSC–FSC plot; right, SSC–CD45 plot). (b) acute myeloid leukaemia bone marrow (left, SSC–FSC plot; right, SSC–CD45 plot). G, granulocytes; M, monocytes; L, lymphocytes; E, erythrocytes; B, blasts. SSC–CD45 gating permits isolation of bone marrow blasts from all other populations, which is not possible by SSC–FSC gating.

8 Confirmation of a diagnosis of multiple myeloma or monoclonal gammopathy of undetermined significance (MGUS), by demonstration that plasma cells (identified by expression of CD138 and strong CD38) have light-chain-restricted cytoplasmic immunoglobulin and often aberrant expression of CD56, together with failure to express CD19 and CD45, and monitoring of MRD (see also Chapter 28).
9 Support for monoclonality of a T-lymphocyte population by demonstration of a uniform aberrant immunophenotype and restricted expression of CD158 (KIR) epitopes and support for specific diagnoses such as T-prolymphocytic leukaemia (CD7 often positive), adult T-cell leukaemia lymphoma (CD25 positive), large granular lymphocytic leukaemia (usually $CD8^+CD4^-$) and hepatosplenic T-cell lymphoma (usually T-cell receptor (TCR)-γδ positive and TCR-αβ negative).
10 Confirmation of a diagnosis of blastic plasmacytoid dendritic cell neoplasm by demonstration of expression of CD4, CD56, CD123 and possibly CD7 in the absence of expression of other lineage-specific markers.
11 Demonstration of an antigen that is a potential target of monoclonal antibody therapy, such as CD20, CD33 or CD52.
12 Demonstration of an antigen that is a potential target of chimaeric antigen receptor T cell (CAR T cell) therapy, such as CD19, CD30, CD33, CD123 and BCMA (B-cell maturation antigen).
13 Diagnosis of paroxysmal nocturnal haemoglobinuria by demonstrating that a subpopulation of neutrophils, monocytes or erythrocytes fails to express antigens, such as CD24, CD14, CD55 and CD59, that are bound to glycosylphosphatidylinositol, or fails to bind proaerolysin conjugated with fluorescein (FLAER).
14 Support for a diagnosis of MDS based on quantification of blasts and detection of aberrant antigen expression.
15 Support for a diagnosis of systemic mastocytosis, with bone marrow infiltration by neoplastic mast cells with aberrant expression of CD25, CD2 and/or CD30.
16 Assessment of infiltration of body fluids (cerebrospinal fluid, pleural effusion) by leukaemia or lymphoma.

Immunohistochemistry

Immunohistochemistry for haematological diagnosis is carried out on tissue sections, particularly from lymph nodes or bone marrow trephine biopsy specimens. It is an essential part of the diagnostic procedure in haematological neoplasms, particularly lymphoma, but also myeloid neoplasms. Examples that illustrate its value in lymphoma diagnosis include the following:
1 Confirmation of follicular lymphoma by demonstration that cells forming neoplastic follicles express CD10 and BCL2 (whereas follicles of reactive follicular hyperplasia are BCL2 negative).
2 Confirmation of mantle cell lymphoma by demonstration of CD5-positive B cells with nuclear expression of cyclin D1 or SOX11.
3 Confirmation of a diagnosis of classical Hodgkin lymphoma (CD15 usually positive, CD30 positive) and its distinction from nodular lymphocyte-predominant Hodgkin lymphoma (CD15 and CD30 usually negative and more likely to express B cell-associated antigens such as CD20 and CD79a).
4 Confirmation of Burkitt lymphoma by demonstration of BCL2-negative BCL6-positive B cells with a very high proliferative fraction (approaching 100%) shown using Ki-67 or an equivalent monoclonal antibody.
5 Division of diffuse large B-cell lymphoma (DLBCL) into prognostic groups (see below).
6 Confirmation of hairy cell leukaemia by showing that B cells are positive for CD25, DBA44 and annexin A1 and express mutant BRAF.
7 Confirmation of clonality in suspected MGUS or multiple myeloma by demonstration of light chain restriction of cytoplasmic immunoglobulin.
8 Confirmation of infiltration in a trephine biopsy specimen in non-Hodgkin and Hodgkin lymphoma (particularly important in patients in whom no neoplastic cells are detected in the bone marrow aspirate and for detection of subtle interstitial or intravascular infiltration in NHL).

DLBCL has been demonstrated by microarray analysis (see below) to be divisible into three major groups with different patterns of gene expression: (i) germinal centre cell; (ii) activated B-cell-like and (iii) other or indeterminate (see also Chapter 27). Lymphomas showing the gene expression pattern of germinal centre B cells were found to have a better prognosis than activated B-cell-like DLBCL (Chapter 27). It is now possible to assign DLBCL to these two major groups on the basis of immunohistochemistry. The germinal centre group express CD10 or, if they do not, they express BCL6 in the absence of MUM1 expression. DLBCL of the activated B-cell-like group are either negative for both CD10 and BCL6 or are CD10 negative, but express both BCL6 and MUM1. The prognostic significance is seen clearly in patients treated with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone), but may no longer reach statistical significance in those treated with R-CHOP (CHOP plus rituximab). In R-CHOP-treated patients, prognostic significance is shown by the demonstration of BCL2 expression, the best prognosis being in those with a germinal centre phenotype, but without BCL2 expression, whose survival curves separate from the other three groups. However, it must be noted that scoring of these immunohistochemical markers is inconsistent between laboratories unless many technical factors are standardised; use of the assignment to germinal centre or non-germinal centre phenotypes to aid treatment decisions would be premature.

In ALL, it is usually possible to perform immunophenotyping on either peripheral blood or a bone marrow aspirate; immunohistochemistry is therefore usually redundant. In AML, immunohistochemistry should be viewed as supplementary to flow cytometric immunophenotyping. It is often

unnecessary. However, in the absence of peripheral blood blast cells and if a bone marrow aspirate cannot be obtained, it becomes essential for diagnosis. It can thus be important for the diagnosis of acute megakaryoblastic leukaemias, and some cases of acute erythroleukaemia, when bone marrow fibrosis may prevent an adequate aspirate from being obtained. It is needed for the diagnosis of myeloid sarcoma and blastic plasmacytoid dendritic cell neoplasm (which shows expression of CD4, CD56 and CD123). Immunohistochemistry can be a useful way to demonstrate the presence of an *NPM1* mutation in AML, cytoplasmic rather than nuclear expression being a surrogate marker for these mutations.

Immunohistochemistry for mast cell tryptase is important in confirming a diagnosis of systemic mastocytosis (Chapter 35). Aberrant expression of CD2 and CD25 by the mast cells is also useful (although this pattern of reactivity is not totally specific for systemic mastocytosis). Immunohistochemistry is much less often needed in other myeloproliferative neoplasms.

Cytogenetic analysis

Cytogenetic analysis refers to study of chromosomes by recognition of their morphology and their characteristic banding pattern, demonstrated by staining with a Giemsa stain (for viewing by light microscopy) (Figure 10.27) or a quinacrine stain (for viewing as fluorescent bands under UV illumination). Chromosomes or specific genes of interest in either interphase or metaphase can also be studied by FISH analysis; this technique is both a cytogenetic and molecular genetic technique since the morphology of the chromosomes can be recognised to some extent, at least in metaphase cells, but the probes used are dependent on hybridisation to specific DNA sequences. Cytogenetic, FISH and molecular techniques are complementary and which of these is most useful in an individual patient depends on the suspected diagnosis and the aberrations likely to be present. Classical cytogenetic analysis (chromosome banding analysis) requires cells in metaphase so that for conditions with a low mitotic rate, such as CLL and low-grade NHL, FISH or molecular analysis may be preferred. However, with the newer techniques, it is possible to achieve metaphases in more than 90% of cases of CLL using specific culture conditions, including DSP30. Cytogenetic analysis can: (i) provide evidence of clonality and thus confirm that a condition is neoplastic when other evidence is absent or equivocal (e.g. in MDS, chronic eosinophilic leukaemia, myeloproliferative neoplasms or NK cell leukaemia/lymphoma), (ii) confirm a specific diagnosis (e.g. CML, AML with recurrent translocations or Burkitt lymphoma) and (iii) give prognostic information (e.g. in CLL, AML, ALL, MDS, CML and other myeloproliferative neoplasms and multiple myeloma (the latter especially by FISH on CD138+ selected cells) or indicate that a neoplasm is therapy-related (t-AML and t-MDS).

It is particularly important that cytogenetic or molecular genetic analysis is performed whenever there is a specific treatment available for patients with a particular genetic abnormality. This includes the following:

1 Conditions sensitive to tyrosine kinase inhibitors (with either *BCR::ABL1,* rearrangement of *PDGFRA*, *PDGFRB, FGFR1* or *PCM1::JAK2*).

2 Acute promyelocytic leukaemia with *PML::RARA* (sensitive to all-*trans* retinoic acid and As_2O_3).

3 MDS with 5q– (sensitive to lenalidomide).

4 MDS with monosomy 7 (sensitive to azacitidine).

5 Burkitt lymphoma with juxtaposition of *MYC* to the immunoglobulin heavy chain, κ or λ locus (responsive to intensive

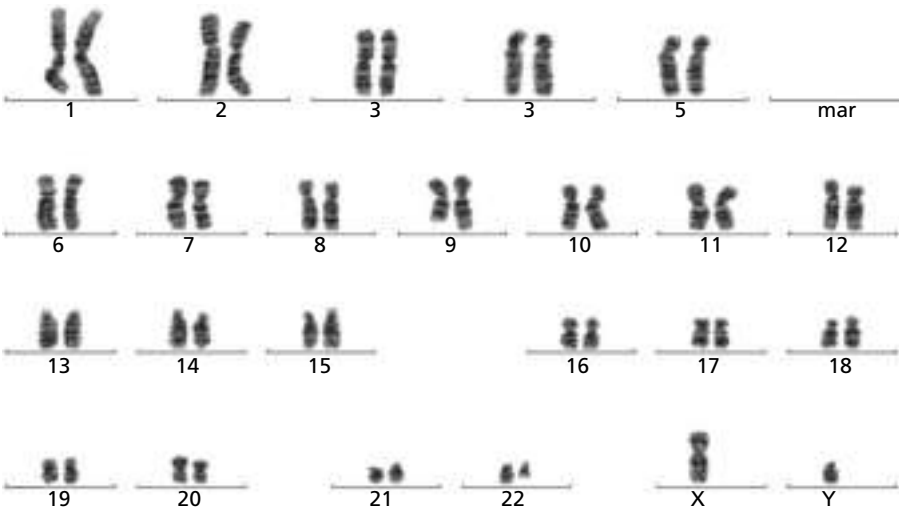

Figure 10.27 A karyogram of Giemsa-stained chromosomes showing t(9;22)(q34.1;q11.2). The derivative chromosome 22 is the Philadelphia chromosome.

Table 10.1 The 2022 WHO classification of acute myeloid leukaemia (AML).

Acute myeloid leukaemia with defining genetic abnormalities*
Acute promyelocytic leukaemia with *PML::RARA* fusion
Acute myeloid leukaemia with *RUNX1::RUNX1T1* fusion
Acute myeloid leukaemia with *CBFB::MYH11* fusion
Acute myeloid leukaemia with *DEK::NUP214* fusion
Acute myeloid leukaemia with *RBM15::MRTFA* fusion
Acute myeloid leukaemia with *BCR::ABL1* fusion†
Acute myeloid leukaemia with *KMT2A* rearrangement
Acute myeloid leukaemia with *MECOM* rearrangement
Acute myeloid leukaemia with *NUP98* rearrangement
Acute myeloid leukaemia with *NPM1* mutation
Acute myeloid leukaemia with *CEBPA* mutation†
Acute myeloid leukaemia, myelodysplasia-related
Acute myeloid leukaemia with other defined genetic alterations
Acute myeloid leukaemia, defined by differentiation
Acute myeloid leukaemia with minimal differentiation
Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia

* Either cytogenetic or molecular genetic analysis is essential for classification.
† AML with *BCR::ABL1* and AML with *CEBPA* mutation are the only disease types with a defined genetic abnormality that require at least 20% blasts for diagnosis.

Table 10.2 The WHO 2022 classification of acute lymphoblastic leukaemia (see also Chapters 21 and 22).

Precursor B-cell neoplasms
B lymphoblastic leukaemia/lymphoma
B lymphoblastic leukaemia/lymphoma, not otherwise specified
B-lymphoblastic leukaemia/lymphoma with high hyperdiploidy
B-lymphoblastic leukaemia/lymphoma with hypodiploidy
B-lymphoblastic leukaemia/lymphoma with iAMP21
B-lymphoblastic leukaemia/lymphoma with *BCR::ABL1* fusion
B-lymphoblastic leukaemia/lymphoma with *BCR::ABL1*-like features
B-lymphoblastic leukaemia/lymphoma with *KMT2A* rearrangement
B-lymphoblastic leukaemia/lymphoma with *ETV6::RUNX1* fusion*
B-lymphoblastic leukaemia/lymphoma with *ETV6::RUNX1*-like features
B-lymphoblastic leukaemia/lymphoma with *TCF3::PBX1* fusion
B-lymphoblastic leukaemia/lymphoma with *IGH::IL3* fusion
B-lymphoblastic leukaemia/lymphoma with *TCF3::HLF* fusion
B-lymphoblastic leukaemia/lymphoma with other defined genetic abnormalities
Precursor T-cell neoplasms
T-lymphoblastic leukaemia/lymphoma
T-lymphoblastic leukaemia /lymphoma, NOS
Early T-precursor lymphoblastic leukaemia/lymphoma

* Molecular genetic analysis or whole transcriptome sequencing is needed since the translocation is cryptic.

chemotherapy incorporating certain specific agents plus monoclonal antibody treatment).

Cytogenetic analysis can be considered appropriate whenever it contributes to diagnosis, indicates the prognosis or guides the selection of treatment. It can therefore be considered indicated in the following:

1 All cases of AML: essential for application of the WHO and ICC classifications, indicate prognosis and is thus relevant to treatment choice (Table 10.1).

2 All cases of ALL: essential for application of the WHO and ICC classifications, indicate prognosis and is thus relevant to treatment choice; necessary for detection of *BCR::ABL1*-positive cases, although for this purpose FISH and molecular analyses are alternatives (Table 10.2).

3 All cases of mixed-phenotype acute leukaemia: essential for detecting *BCR::ABL1*-positive cases.

4 All cases of suspected CML: essential to confirm *BCR::ABL1* and to identify additional chromosomal aberrations of prognostic relevance

5 All cases of suspected MDS: necessary for application of the WHO and ICC classifications, including identification of the 5q– syndrome (Figure 10.28) and essential for application of the revised International Prognostic Scoring System (IPSS-R) and WHO classification-based Prognostic Scoring System (WPSS).

6 Selected cases of myeloproliferative neoplasms to identify a clonal marker and to allow prognostic risk stratification (see Chapter 35).

7 All cases of suspected chronic eosinophilic leukaemia: necessary for detection of translocations that lead to *PDGFRB* rearrangement (imatinib sensitive) and *FGFR1* rearrangement.

8 All cases of suspected NK leukaemia/lymphoma: can demonstrate clonality and thus confirm the diagnosis.

If resources permit, cytogenetic analysis can also be useful in other myeloproliferative and myelodysplastic/myeloproliferative neoplasms. Cytogenetic analysis can be used for the confirmation of a diagnosis of Burkitt lymphoma, follicular lymphoma or mantle cell lymphoma, but FISH may be preferred. Use of the two techniques may give complementary information. Prognostically relevant information is also obtained in multiple myeloma. However, this is best done with

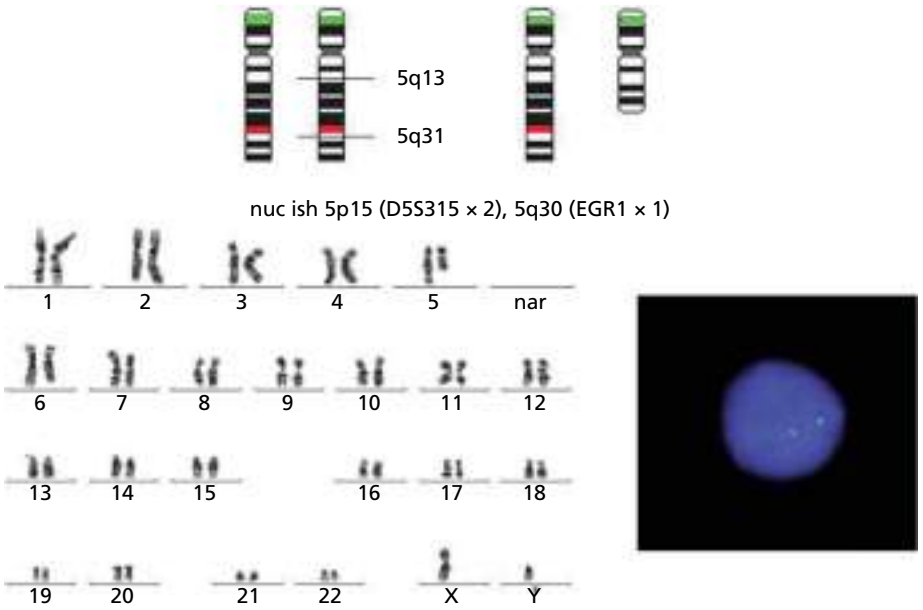

Figure 10.28 An explanatory diagram (top), karyogram of Giemsa-stained chromosomes showing del(5)(q13q31) (bottom left), and FISH showing retention of signal for 5p15 and loss of signal for 5q31 (bottom right) in a patient with 5q– syndrome.

preliminary magnetic activation cell sorting using CD138, CD269 or CD319 monoclonal antibodies, as plasma cells numbers may be low and FISH performed on direct bone marrow films may result in insufficient cells for analysis and give misleading results.

Fluorescence *in situ* hybridisation

FISH uses labelled oligonucleotide probes that bind to specific DNA sequences. These may be locus-specific probes (including those detecting oncogenes and tumour-suppressor genes), centromeric probes, telomeric probes and whole chromosome paints. Centromeric probes can be used for the detection of monosomies and trisomies. Otherwise, locus-specific probes are the most widely used. By labelling two probes with different fluorochromes, it is possible to study two genes that are involved in a specific translocation or other rearrangement. If the probes span the breakpoint, both will be disrupted by the translocation and signals will be adjacent or optically fused on the derivative chromosomes, a double-colour double-fusion technique. Often one probe gives a green signal and the other a red signal so that the fusion signal is yellow. Alternatively, two probes that span the 5′ locus and 3′ locus and overlap can be used in a double-colour break-apart technique; this can be useful when a single gene, such as *KMT2A*, is involved in rearrangements with a large number of other genes.

FISH is applicable to both metaphase cells and interphase cells (Figure 10.29). FISH is particularly useful in the following circumstances:

1 Screening for *BCR::ABL1* fusion (peripheral blood cells are suitable, whereas cytogenetic analysis is better done on bone marrow) (Figure 10.30).
2 Confirming a specific lymphoma diagnosis by demonstration of *BCL2::IGH* in follicular lymphoma, *CCND1::IGH* in mantle cell lymphoma or *MYC::IGH* in Burkitt lymphoma (Table 10.3).
3 Detection of translocations or inversions or aneuploidy that was not detected on metaphase cytogenetic analyses because the leukaemic cells did not enter mitosis or the metaphases were of too poor quality, e.g. detection of t(8;21)(q22;q22.1); *RUNX1::RUNX1T1*, t(15;17)(q24.1;q21.1); *PML::RARA*, inv(16)(p13.1q22); *CBFB::MHY11* or *KMT2A* rearrangement in AML (Figure 10.31) or detection, in ALL, of iAMP21 (using a *RUNX1* probe) or high hyperdiploidy (by using centromeric probes for the chromosomes that are most often triplicated).
4 Screening for a cryptic *FIP1L1::PDGFRA* fusion (by detection of loss of *CHIC2*) in myeloid/lymphoid neoplasm with *PDGFRA* rearrangement (including chronic eosinophilic leukaemia).
5 Producing prognostically relevant information in CLL by investigation for trisomy 12, del(6)(q21), del(11)(q22–23), del(13)(q14.3) (Figure 10.32) and del(17)(p13).

Spectral karyotyping, also known as 24-colour FISH, is a modification of FISH that permits visualisation of each pair of chromosomes in a unique colour. It can be used as a supplement to metaphase cytogenetics to clarify the nature of complex rearrangements (Figure 10.33). Inversions are not detected.

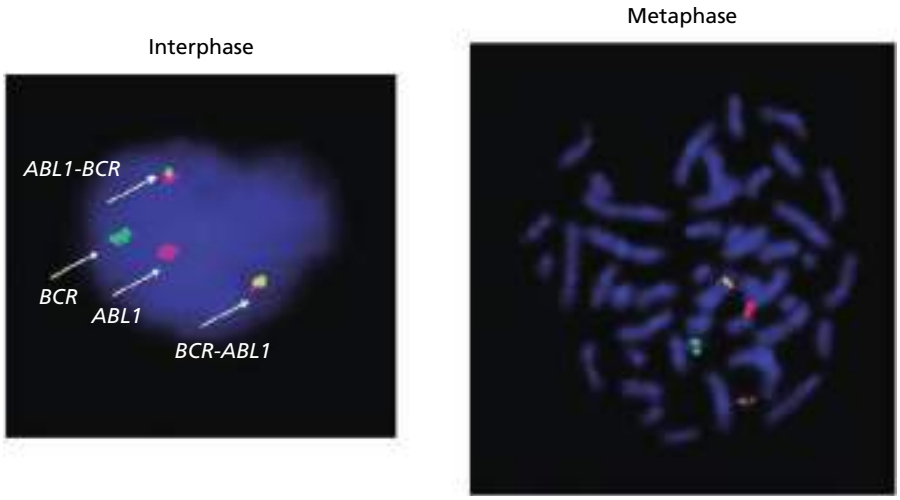

Figure 10.29 Locus-specific probes for *BCR* (green) and *ABL1* (red) applied to an interphase cell and to a metaphase of a patient with chronic myeloid leukaemia after hybridisation with probes for *ABL1* (red) and *BCR* (green) showing *BCR::ABL1* co-localisation signals (yellow) on the Philadelphia chromosome and *ABL1::BCR* co-localisation signals on derivative chromosome 9.

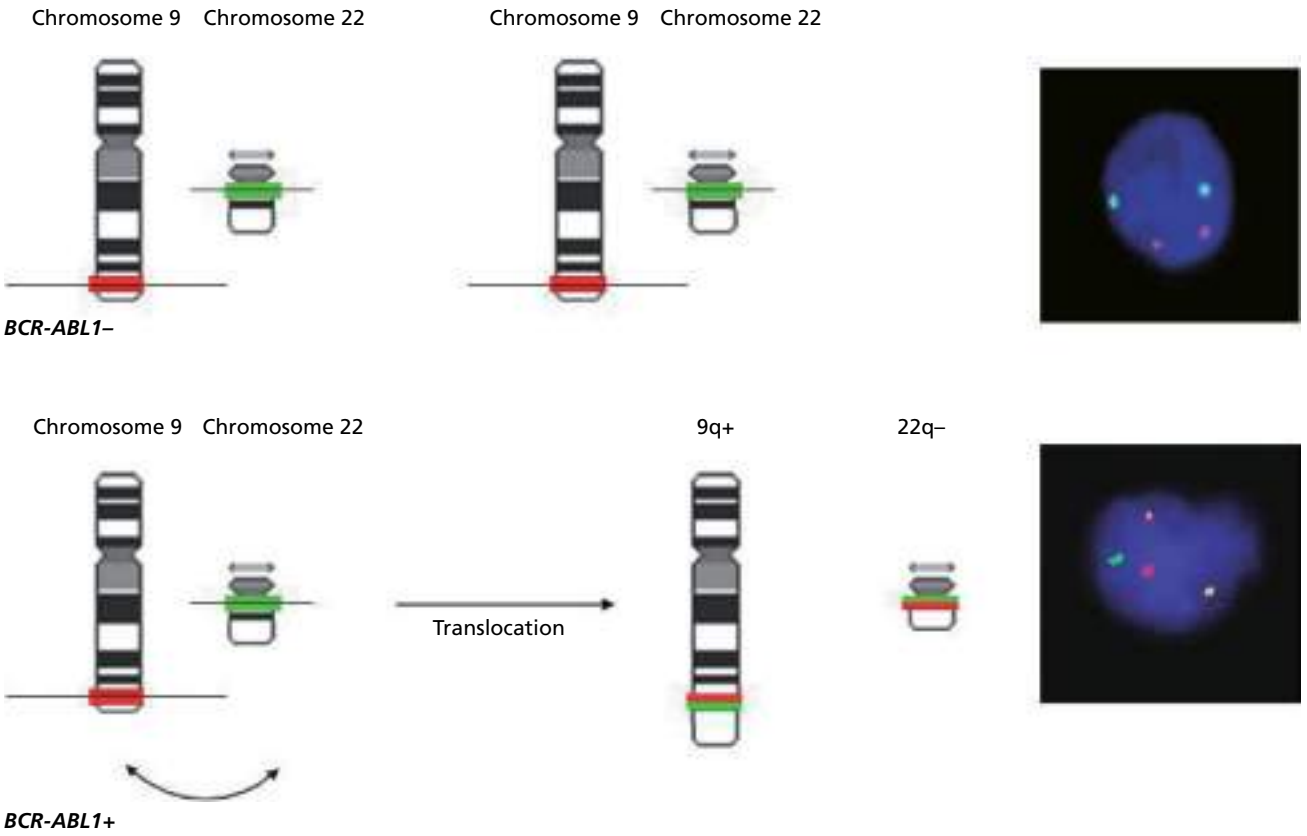

Figure 10.30 Diagram showing the principle of fluorescence *in situ* hybridisation for detection of *BCR::ABL1*. Locus-specific probes for *BCR* (green) and *ABL1* (red) have been applied to interphase cells. The normal cell (top) shows two red signals and two green signals, whereas the cell with *BCR::ABL1* shows two normal signals and two fusion signals (*BCR::ABL1* and *ABL1::BCR*).

Table 10.3 Cytogenetic/molecular genetic abnormalities that were incorporated into the 2016 WHO classification of neoplasms of mature T and B cells.

Genetic abnormality	Gene dysregulated	Diagnosis*
t(14;18)(q32;q21) or t(2;18)(p12;q21) or t(18;22)(q21;q11)	*BCL2*	Follicular lymphoma
t(11;14)(q13;q32) or, rarely, t(11;22)(q13;q11)	*CCND1*	Mantle cell lymphoma
t(8;14)(q24;q32) or t(2;8)(p12;q24) or t(8;22)(q24;q11.2)	*MYC*	Burkitt lymphoma
11q23 gain and 11q24.1-ter loss		Burkitt-like lymphoma with 11q aberration
inv(14)(q11.2q32.1) or t(14;14)(q11.2;q32.1) or, less often, t(X;14)(q28;q11.2)	*TCL1*	T-prolymphocytic leukaemia
t(2;5)(p23.2;q35.1) or one of at least eight variant translocations with a 2p23.2 breakpoint	*NPM1*::*ALK* fusion or other fusion gene incorporating part of *ALK*	Anaplastic large-cell lymphoma, ALK-positive

* The genetic abnormality confirms the diagnosis only in an appropriate cytological/histological and immunophenotypic setting.

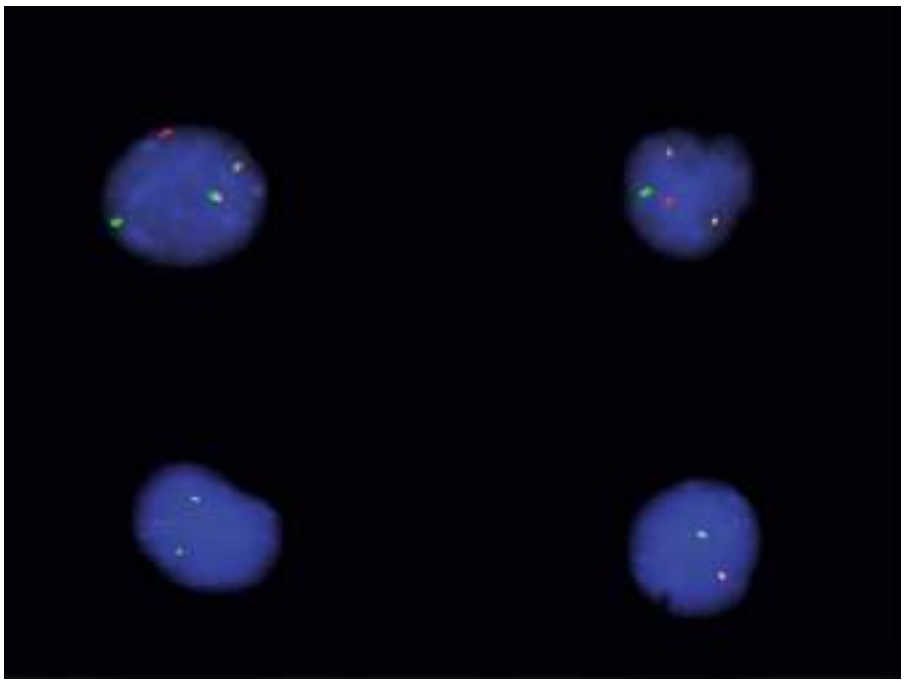

Figure 10.31 Fluorescence *in situ* hybridisation in four different patients with acute myeloid leukaemia showing four major cytogenetic/genetic categories of AML: *RUNX1*::*RUNX1T1* fusion indicative of t(8;21) (top left); *PML*::*RARA* fusion indicative of t(15;17) (top right); *CBFB* rearrangement, which is likely to indicate *CBFB*::*MYH11* (bottom left); *KMT2A* rearrangement (bottom right).

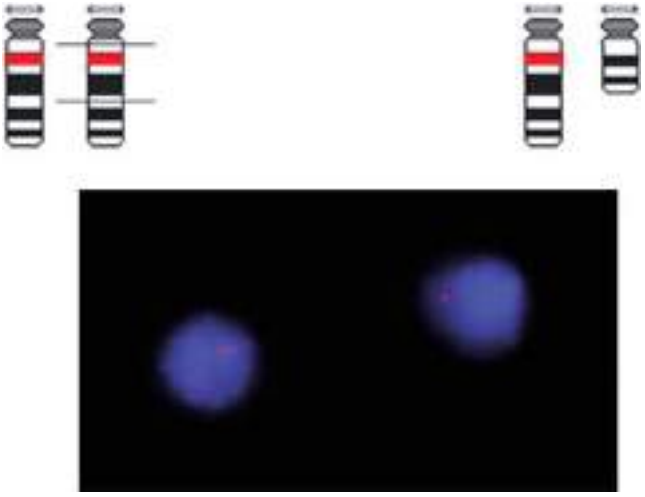

Figure 10.32 Explanatory diagram and fluorescence *in situ* hybridisation showing interstitial deletion of 13q14.3 in a patient with chronic lymphocytic leukaemia.

Molecular genetic analysis

Molecular genetic analysis includes the polymerase chain reaction (PCR) to study genomic DNA (Figures 10.34 and 10.35) and reverse transcriptase polymerase chain reaction (RT-PCR) to study RNA after its reverse transcription. A PCR reaction can also be made quantitative, as in real-time quantitative PCR, an important technique for monitoring MRD (Figure 10.36). PCR and RT-PCR can be alternatives to metaphase cytogenetic analysis in several subgroups of leukaemia and lymphoma. However, unlike metaphase cytogenetic analysis but similar to FISH, this is a targeted investigation so that only the abnormality that is specifically sought will be detected. Thus, in some diseases, additional diagnostic or prognostic information will be found only by using metaphase cytogenetics. On the other hand, PCR has the advantage that only a small amount of DNA is needed and there is no need for dividing cells. Molecular analysis permits the detection of prognostically and therapeutically relevant cryptic rearrangements, such as the *ETV6::RUNX1* rearrangement associated with a cryptic t(12;21)(p13.2;q22.1) in ALL. Genomic PCR can be applied to stored samples.

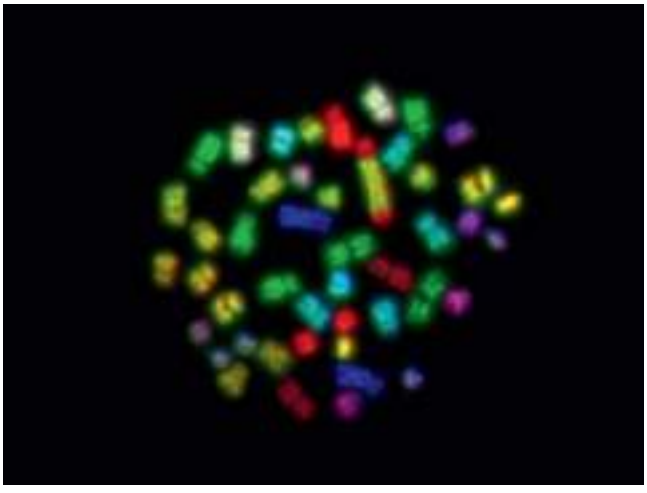

Figure 10.33 Metaphase of a patient with acute myeloid leukaemia and a complex aberrant karyotype after 24-colour fluorescence *in situ* hybridisation.

The roles of genomic PCR and RT-PCR include the following:

1 Detection of rearrangement of immunoglobulin heavy and light chain loci and *TCR* loci, providing evidence of a clonal disorder if a monoclonal rather than an oligoclonal or polyclonal pattern is detected.

2 Detection of leukaemia-related and lymphoma-related chromosomal rearrangements by demonstration of gene juxtaposition or fusion in order to confirm a diagnosis, for example detection of *CCND1::IGH* indicative of t(11;14)(q13;q32) to confirm suspected mantle cell lymphoma or detection of *BCL2::IGH* indicative of t(14;18)(q32;q21) to confirm suspected follicular lymphoma. Note that neither of these rearrangements is absolutely specific for the disease in question since *CCND1::IGH* also occurs in multiple myeloma and *BCL2::IGH* also occurs in DLBCL, but in the context of appropriate cytology or histology they do permit confirmation of a diagnosis.

3 Detection of gene mutations relevant to diagnosis, for example *KIT* D816V in systemic mastocytosis or either *JAK2* V617F or *JAK2* exon 12 mutation in polycythaemia vera. *JAK2* V617F also occurs in many patients with essential thrombocythaemia or primary myelofibrosis, but its detection in a patient with a high haemoglobin concentration confirms the diagnosis of polycythaemia vera and since it is present in the great majority of patients with this diagnosis, measurement of the total red cell mass and plasma volume is generally rendered unnecessary. Patients with suspected essential thrombocythaemia or primary myelofibrosis who are *JAK2* V617F-negative should be further investigated for *CALR* or *MPL* mutation.

4 Prognostic stratification, for example in ALL detection of *ETV6::RUNX1* associated with cryptic t(12;21)(p13.2;q22.1) (good prognosis), *TCF3::PBX1* associated with t(1;19)(q23;p13.3) (good prognosis), *KMT2A::MLLT2* associated with t(4;11)(q21;q23.3) (poor prognosis) and *BCR::ABL1* associated with t(9;22)(q34.1;q11.2) (intermediate prognosis); multiplex PCR, combining a number of primer pairs in a single reaction, is useful for screening simultaneously for more than one rearrangement.

5 Prognostic stratification in AML with normal karyotype, for example *FLT3* internal tandem duplication (ITD) (poor prognosis, depending on allele burden), *KMT2A* partial tandem duplication (PTD) (poor prognosis), *NPM1* mutation (good prognosis if not associated with *FLT3*-ITD), *CEBPA* mutation (good prognosis if biallelic or bZIP region mutated and not associated with *FLT3*-ITD) (see also Chapter 32).

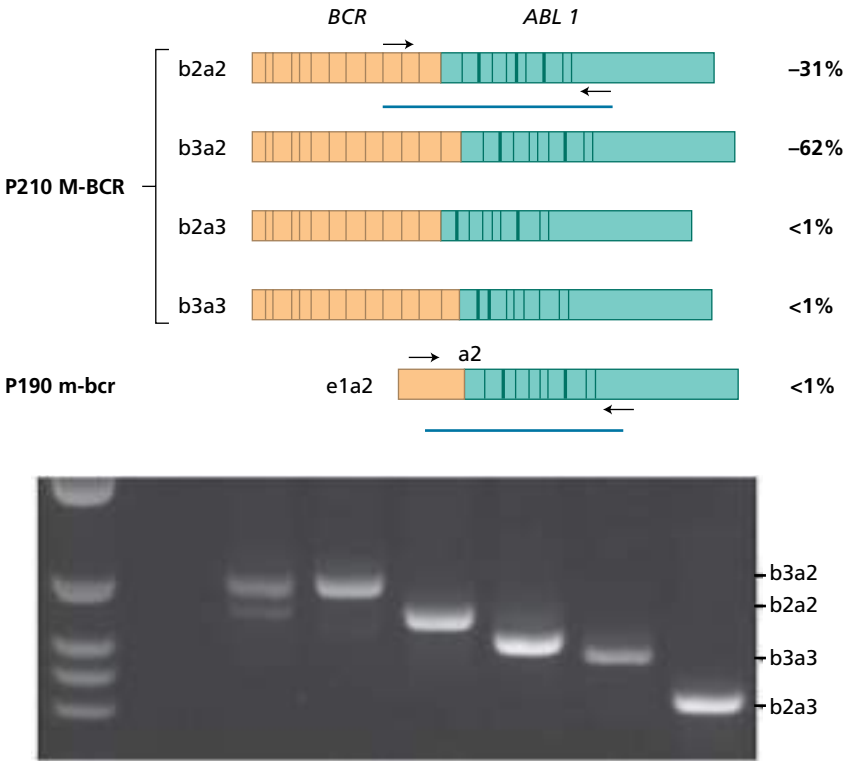

Figure 10.34 An explanatory diagram and a PCR gel showing the various *BCR::ABL1* transcripts that can be identified by PCR.

6 Prognostic stratification in CLL: somatic hypermutation, defined as *IGHV* showing less than 98% homology with germline, is found in 50–60% of all cases and correlates with a better prognosis than is found in patients with unmutated *IGHV* genes. *TP53* mutations are prognostically adverse and guide treatment.

7 MRD detection using *RUNX1::RUNX1T1*, *CBFB::MYH11*, *PML::RARA*, *KMT2A* rearrangement, *KMT2A*-PTD, *FLT3*-ITD, *NPM1* mutation or *CEBPA* mutation.

Whole-genome scanning

There are a number of molecular techniques available for whole-genome scanning. Thus far, these have mainly been used to address research topics, but they are now increasingly finding a place in routine diagnostic work. The NCCN guidelines 3/2023 in AML for the first time include whole genome sequencing (WGS) in the diagnostic approach to AML. These

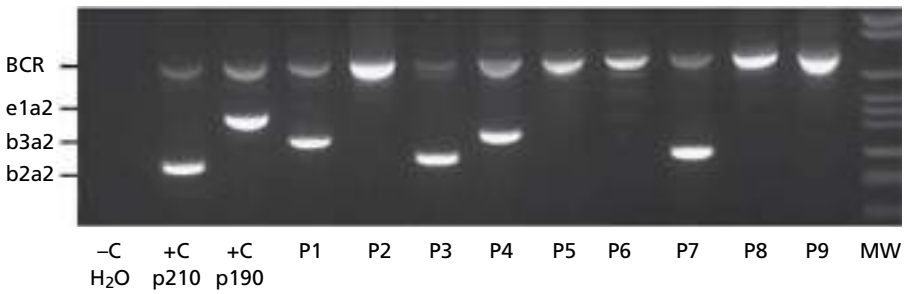

Figure 10.35 *BCR::ABL1* multiplex PCR containing different oligonucleotide ('oligo') combinations. –C: negative control sample containing water instead of a nucleic acid template; +C: positive controls for p210 and p190 fusion types; P: patient samples, P1 and P4 are positive for b3a2 fusion types, P3 and P7 are positive for b2a2 fusion types, P2, P5, P6, P8 and P9 are *BCR::ABL1*-negative patient samples; MW, molecular weight standard; BCR, amplification of the non-rearranged *BCR* gene as internal quality control reaction.

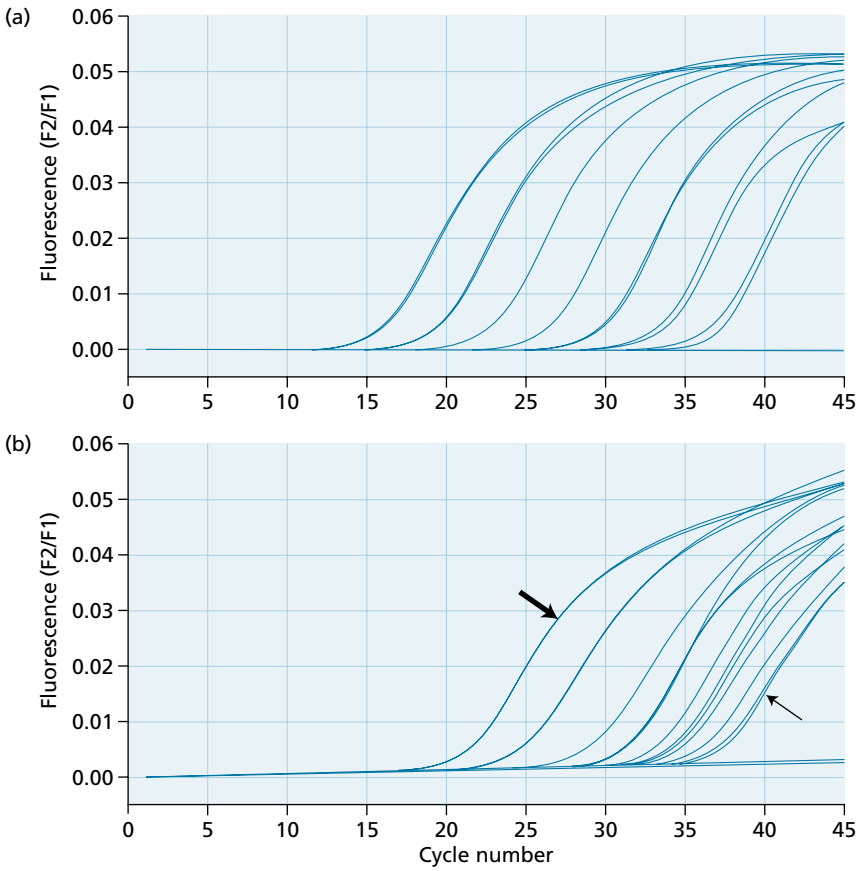

Figure 10.36 Real-time quantitative PCR. Fluorescence is plotted against PCR cycle number. (a) A 10-fold limited dilution series of a *BCR::ABL1*-containing plasmid showing high sensitivity of the PCR. It also serves as a calibration curve for subsequent *BCR::ABL1* calculations. (b) Run with chronic myeloid leukaemia follow-up samples: the large arrow shows a sample with high *BCR::ABL1* load and the small arrow low *BCR::ABL1* load.

techniques include comparative genomic hybridisation (CGH), in which labelled test DNA is hybridised to normal metaphase preparations, and microarray analysis, in which there is hybridisation of the labelled test DNA of interest to probes positioned in high density on a microarray surface. Microarray analysis includes CGH arrays and, more frequently used, genome-wide single-nucleotide polymorphism (SNP) arrays.

In CGH assays, test and reference DNAs are labelled with different fluorochromes before being hybridised to microarrays of either oligonucleotides or bacterial artificial chromosome (BAC) probes. CGH microarray analysis is used for the detection of under-representation or over-representation of specific genomic regions in the test DNA. Deletions and amplifications can thus be detected, for example deletion of 13q14 and 11q22 in CLL or iAMP21 in ALL.

In SNP array analysis, genomic DNA of a sample of interest is digested by restriction enzymes, amplified, labelled and hybridised to oligonucleotide probes. Probes are either bound to microbeads or are synthesised *in situ* in high density on a microarray surface (Figure 10.37). SNP analysis can be applied to genotyping and is able to distinguish heterozygosity from homozygosity. It can thus be used to detect both loss of heterozygosity and alterations of copy numbers of a genomic region (i.e. gains and losses). Acquired somatic uniparental disomy can be detected by comparison of tumour DNA with constitutional DNA. Sensitivity for the detection of a clonal population is similar to that of metaphase cytogenetics. With SNP array technology, it is possible to identify uniparental disomy for chromosomal regions that include mutated genes such as *JAK2*, *CEBPA*, *FLT3* and *RUNX1*. SNP array analysis does not typically detect chromosomal translocations.

Microarray analysis of gene expression

Microarray analysis was initially applied to the study of cellular gene expression by hybridisation of test and control RNA specimens, processed and labelled with different fluorochromes, to single-stranded DNA. The DNA probes were either complementary (c) DNA or synthetic oligonucleotides, arrayed in multiple rows of dots on a microarray surface with each dot interrogating a fragment of a single gene. High-density gene expression profiling (GEP) is a well-established molecular method and has been demonstrated to yield information of both diagnostic and prognostic value.

Primary mediastinal B-cell lymphoma can be distinguished from both DLBCL and Hodgkin lymphoma by its molecular signature. Burkitt lymphoma has a distinctive pattern of gene expression, which can help in making a distinction from DLBCL with *MYC* over-expression. Similarly, small lymphocytic lymphoma,

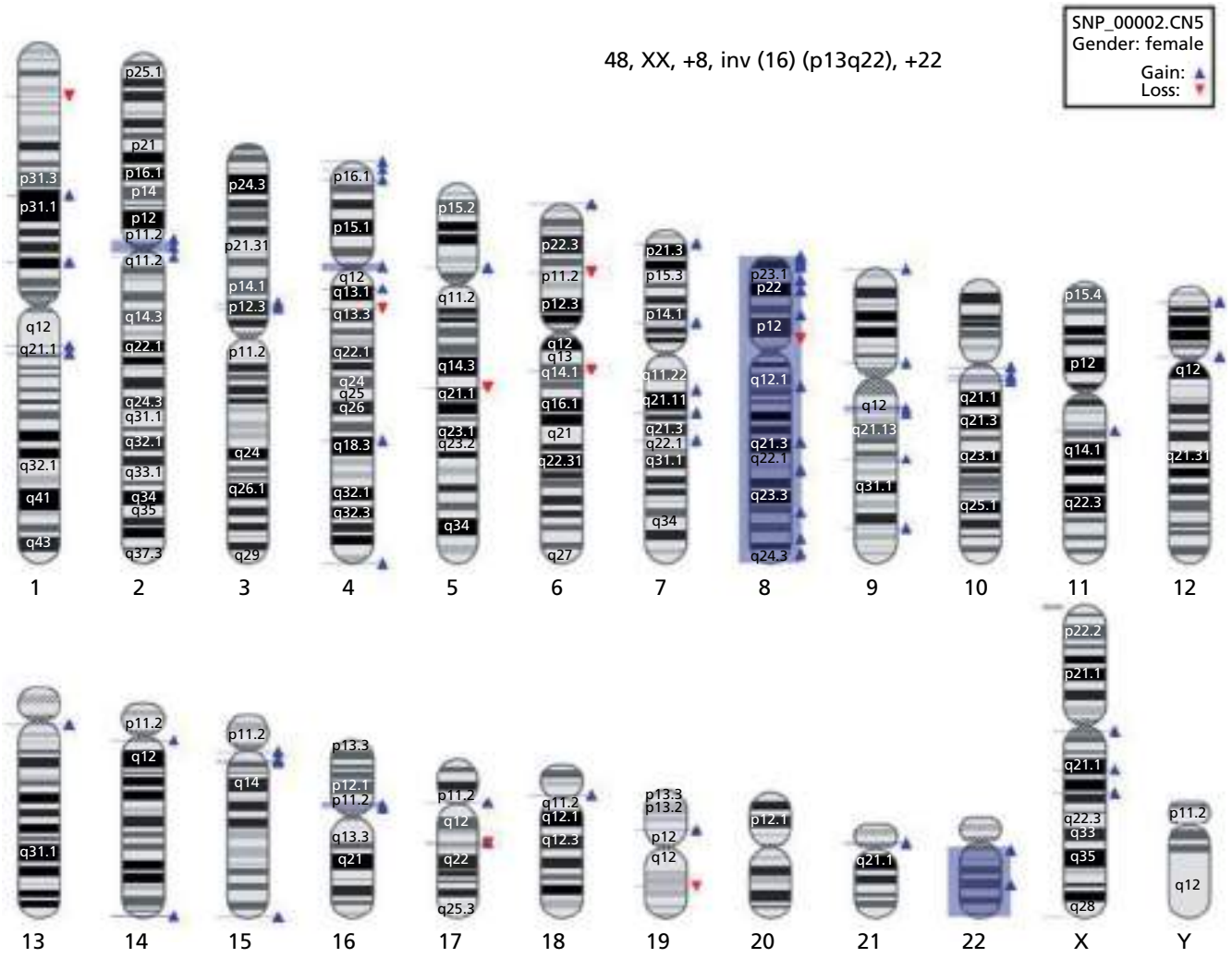

Figure 10.37 Molecular karyotype based on high-density SNP microarray analysis. Alterations are given by arrows indicating either gains or losses of chromosomal material. The karyotype using conventional chromosome banding techniques is given for comparison.

marginal zone B-cell lymphoma and mantle cell lymphoma can be characterised by their different gene expression signatures.

Prognostic information is provided in DLBCL. Microarray studies have shown that this diagnostic category encompasses at least two molecularly distinct diseases, differing in differentiation stage (i.e. cell of origin), oncogenic mechanisms and clinical outcome. Microarray analysis can also divide mantle cell lymphoma into groups with very different prognoses.

Next-generation sequencing

The constantly increasing spectrum of molecular mutations is a tremendous challenge for haematological laboratories. Thus, the introduction of high-throughput sequencing technologies, which allow the massive parallel analysis of hundreds of thousands of alleles in a short time, provides new options for molecular mutation analyses and for follow-up diagnostics in haematological neoplasms. Today, panel sequencing is already in routine use in many laboratories and whole exome sequencing (WES), whole genome sequencing (WGS) and whole transcriptome sequencing (WTS) are already in clinical use. These techniques and their application are discussed in detail in Chapter 8.

Laboratory techniques and the WHO classification of tumours of haemopoietic and lymphoid tissues

The 2022 WHO classification is based on the integration of clinical, pathological and molecular features. Amongst the pathological features, morphology (cytology and histology) is fundamental, but there is an increasing use of immunophenotyping for classification of lymphoid, mixed lineage and

some myeloid neoplasms and of cytogenetic and molecular genetic information for classification of both lymphoid and myeloid neoplasms. Tables 10.1 and 10.2 illustrate how cytogenetic and molecular genetic information is integrated into the classifications of AML and B-lineage ALL respectively. Two categories of acute leukaemia of ambiguous lineage are also defined by the presence, in addition to the required immunophenotypic characteristics, of t(9;22)(q34.1;q11.2); *BCR::ABL1* and 11q23/*KMT2A* rearrangement respectively. The 2022 WHO classification includes genetically defined categorisation for lymphoid and myeloid neoplasms, for example rearrangement of *PDGFRA*, *PDGFRB*, *FGFR1, JAK2, FLT3* and *ETV6::ABL1* as genetic abnormalities defining myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions. Otherwise, amongst the myeloproliferative neoplasms, only the demonstration of t(9;22)(q34.1;q11.2); *BCR::ABL1* to confirm or exclude a diagnosis of CML is essential for classification. However, the demonstration of the *JAK2* V617F mutation or a somatic *MPL* or *CALR* mutation is important for making a diagnosis of a myeloproliferative neoplasm rather than a secondary or familial disorder. A *JAK2* exon 12 mutation similarly confirms that polycythaemia is polycythaemia vera rather than a familial or secondary condition.

Cytogenetic and molecular genetic abnormalities are critical in the diagnosis of certain neoplasms of mature lymphocytes (Table 10.3). However, for the majority of cases of CLL and NHL, such analysis is not needed for diagnosis and classification, although it may provide information of prognostic value.

Conclusions

Modern diagnosis, classification and monitoring of haematological neoplasms require integration of multiple diagnostic tools in a systematic manner in order to provide all the information necessary for optimal management of the patient. In addition, application of new diagnostic tools is constantly increasing our understanding of these disorders. New research tools not only give new information but are often rapidly integrated into diagnostic practice. Classifications of lymphoid and myeloid neoplasms increasingly incorporate and depend on immunophenotypic and genetic analysis. High-throughput sequencing technologies investigating gene panels by next-generation sequencing have now been introduced into routine diagnostic algorithms. WES, WGS and WTS are also becoming routinely available in laboratory diagnostics and in certain settings look set to replace other more traditional diagnostic techniques over the coming years. Especially in ALL, some entities such as ALL with *DUX4* overexpression need WTS for diagnosis.

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PART II

Red blood cell disorders and blood transfusion

CHAPTER 11

Iron metabolism, iron deficiency and disorders of haem synthesis

11

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Introduction

Iron is essential for many metabolic processes. It shares with other transition metals two properties of particular importance in biology: the ability to exist in more than one relatively stable oxidation state and the ability to form many complexes. Its ability to exist in both ferric and ferrous states underlies its role in critical enzyme reactions concerned with oxygen and electron transport and the cellular production of energy. As well as physiologically active iron compounds, many of which are haem proteins, there are also specialised proteins of iron absorption, transport and storage. The latter are necessary to enable iron to remain in solution at neutral pH, at which ferric iron is insoluble, and to limit the potential toxicity of this reactive metal. The insolubility of ferric iron also means that although the Earth's crust contains approximately 4% iron and iron may be plentiful in the diet, much of this is not bioavailable. As a result, the body is limited in the adjustments it can make to excessive loss of iron, which frequently occurs due to haemorrhage, and iron deficiency is the most common cause of anaemia throughout the world, affecting in the order of 1 billion people. The general need to conserve the metal is reflected in the absence of any physiological mechanism for excretion of iron, control of iron balance being at the level of iron absorption. This is important in the rarer, but potentially fatal disorders of iron overload (see Chapter 12).

Distribution of body iron

The amount of iron in the adult human body is normally about 50 mg/kg in males and 40 mg/kg in females. The largest component is circulating haemoglobin, with 450 mL (1 unit) of whole blood containing about 200 mg of iron. Much of the remainder is contained in the storage proteins ferritin and haemosiderin. These are found mainly in the macrophages of the liver, spleen and bone marrow (which gain iron from breaking down red cells), and in parenchymal liver cells (which normally obtain most of their iron from the plasma iron-transporting protein transferrin) (Figure 11.1).

Proteins important in iron metabolism

Haem proteins and iron-containing enzymes

Haemoglobin contains four haem groups linked to four globin chains and can bind four molecules of oxygen. Myoglobin accounts for 4–5% of body iron and has a single haem group attached to its one polypeptide chain. It has a higher affinity for oxygen than haemoglobin and behaves as an oxygen reserve in muscles. The mitochondria contain a series of haem and non-haem iron proteins (including the cytochromes *a*, *b* and *c*, succinate dehydrogenase and cytochrome oxidase) that form an electron transport pathway responsible for the oxidation of

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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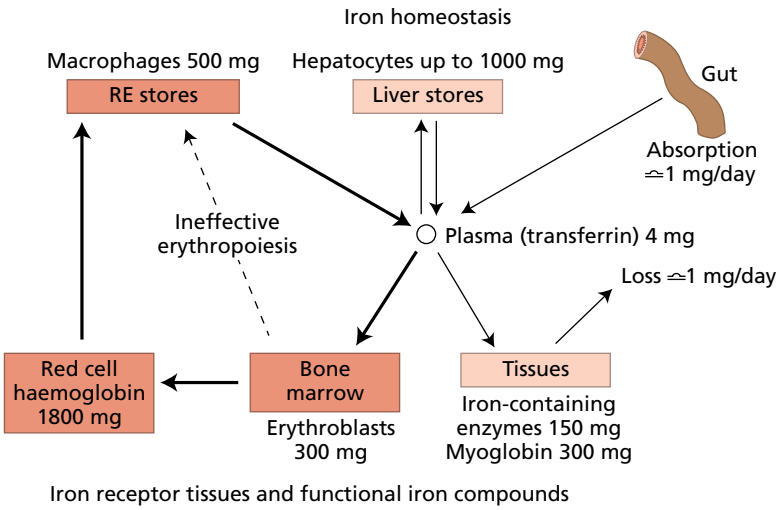


Figure 11.1 Iron homeostasis. The major compartments of iron in a 70-kg man. Iron supply for erythropoiesis and release of iron from senescent red cells dominate internal iron exchange. RE, reticuloendothelial. The dotted line indicates the low proportion of iron that derives from ineffective erythropoiesis in normal subjects.

intracellular substrates and the simultaneous production of adenosine triphosphate (ATP). Haem is an essential component of microsomal and mitochondrial cytochrome P450, which is concerned with hydroxylation reactions (including drug detoxification by the liver), and of cyclooxygenase, involved in prostaglandin synthesis. Other haem proteins include the enzymes catalase and lactoperoxidase, which are concerned with peroxide breakdown, and tryptophan pyrrolase, involved in the oxidation of tryptophan to formylkynurenine. There is a smaller but biologically important group of iron–sulfur proteins (e.g. xanthine oxidase, reduced nicotinamide adenine dinucleotide dehydrogenase and aconitase). Iron is also necessary for the function of ribonucleotide reductase, a key enzyme in DNA synthesis

Ferritin and haemosiderin

Ferritin is the primary iron storage protein and provides a reserve of iron. It consists of an approximately spherical apoprotein shell enclosing a core of ferric hydroxyphosphate (up to 4000 iron atoms). Human ferritin is made up of 24 subunits of two immunologically distinct types: H and L. There are multiple gene copies, which are mostly pseudogenes. An intronless gene codes for mitochondrial ferritin, an H-type ferritin. The internal cavity of the ferritin molecule communicates with the exterior via six channels, through which ferrous iron may enter (to interact with a ferroxidase centre on the ferritin H subunit) or leave (after reduction, e.g. by dihydroflavins or ascorbic acid).

Ferritin iron is mobilised by ferritinophagy, a process mediated by nuclear receptor co-activator 4 (NCOA4), which, degraded by proteasome in iron-replete cells, binds and addresses ferritin to lysosome degradation in iron deficiency thereby recovering intracellular iron. Variation in the proportion of H to L subunits explains the heterogeneity of ferritin from different tissues on isoelectric focusing: L-rich ferritins (from spleen and liver) are more basic than H-rich ferritins (from heart and red cells). The small amount of ferritin normally present in serum contains little iron and consists almost exclusively of L subunits. It is also heterogeneous, owing to glycosylation. This glycosylation and the direct relationship of serum concentration to storage of iron in macrophages suggest that serum ferritin is secreted by macrophages in response to changing iron levels.

Haemosiderin, unlike ferritin, is a water-insoluble, crystalline, protein–iron complex that is visible by light microscopy when stained by the Prussian blue (Perls') reaction. It has an amorphous structure, with a higher iron/protein ratio than ferritin, and is probably formed by the partial digestion of ferritin aggregates by lysosomal enzymes. In normal subjects, the majority of storage iron is present as ferritin, and haemosiderin is predominantly found in macrophages rather than hepatocytes. In iron overload, the proportion present as haemosiderin increases considerably in both cell types.

Transferrin and transferrin receptors

Transferrin is a single-chain polypeptide present in plasma (1.8–2.6 g/L) and extravascular fluid (Table 11.1). It has a plasma half-life of 8–11 days. The protein is synthesised predominantly by the liver, synthesis being inversely related to iron stores. Two atoms of ferric iron can bind to each molecule. Although transferrin contains only about 4 mg of body iron at any time, it is vital for iron transport, with over 30 mg of iron passing through this compartment each day (Figure 11.1). The uptake of iron from transferrin requires that the protein is attached to specific receptors on the cell surface. The transferrin receptor gene (*TFRC*) codes for TFR1 a transmembrane protein (identified as CD71). Each molecule of two subunits binds with high affinity one molecule of diferric (two iron molecules bound) transferrin.

A truncated form of TFR1 corresponding to the ectodomain is found in serum. This soluble TFR reflects the density of the

Table 11.1 Iron transport proteins, oxidoreductases, storage proteins and regulators.

Protein (gene)	Chromosome location	Tissue expression	Structure	Function	Regulation	Mutations and disease
ATP binding cassette superfamily member 7 (*ABCB7*)	Xq13.13	Ubiquitous, mitochondrial	Protein of the mitochondrial inner membrane	Fe/S export, complex with ABCB10 and ferrochelatase	Unknown	Sideroblastic anaemia with Ataxia
ATP binding cassette superfamily member 10 *(ABCB10)*	1q42.13	Ubiquitous, mitochondrial	Protein of the mitochondrial inner membrane	Interacts with mitoferrin1 and ABCB7 for haem synthesis	E2F4 transcription factor	Knock out mouse has haem defect
Divalent metal transporter 1, DMT1 (*SLC11A2*)	12q13	Widespread	TMP, several isoforms	Fe uptake, endosome transporter	Fe (3′ IRE) HIF-2α	*Mk* mouse, *Belgrade* rat Human microcytic anaemia
Duodenal cytochrome b_1 (*CYBRD1*)	2q31	Duodenal enterocytes	TMP, 6TMD	Ferric reductase	HIF-2α	Not known
Erythroferrone (*ERFE*)	2q37.3	Erythroid precursors, muscle	Secreted protein	Hepcidin inhibitor Sequesters BMPs	Erythropoietin	Somatic mutation in MDS RS
Feline leukaemia virus C receptor (*FLVCR1*)	1q32.3	Erythroid, GI tract, liver, endocrine tissue and muscle	Major facilitator Family	Receptor for feline leukaemia virus C; haem export	Not known	Not known
Ferritin light chain (*FTL*)	19q13.3	Widespread, cytosolic	Subunit of ferritin	Facilitates iron storage	Fe (5′ IRE)	Hyperferritinaemia-cataract syndrome. Neuroferritinopathy*
Ferritin heavy chain *(FTH)*	11q13	Widespread, cytosolic	Subunit of ferritin	Iron storage: catalytic subunit for iron incorporation	Fe (5′ IRE)	Not known
Ferroportin (*SLC11A3*)	2q32	Liver, spleen, enterocytes, erythrocytes and trophoblasts	TMP, 9TMD	Fe export	Fe (5′-IRE) Cytokines Hepcidin	Ferroportin disease: *loss of function* mutations autosomal dominant HC: *gain of function* mutations

(*Continued*)

Table 11.1 (*Continued*)

Protein (gene)	Chromosome location	Tissue expression	Structure	Function	Regulation	Mutations and disease
Frataxin (*FXN*)	9q21.11	Heart, spinal cord and cerebellum	Mitochondrial protein	Mitochondrial iron donor	?	Friedreich ataxia
Glutaredoxin 5 *(GLRX5)*	14q32.13	Widespread, mitochondrial	Mitochondrial enzyme	Fe/S clusters transfer		Sideroblastic anaemia
Haemochromatosis (*HFE*)	6p21.3	Widespread	HLA class I heavy chain	Regulates cell iron uptake and hepcidin expression	?	Human HC, autosomal recessive
Hemojuvelin (*HJV*)	1q21.2	Liver, heart and muscle	Membrane-bound BMP coreceptor	Regulator of hepcidin synthesis	TMPRSS6 (posttranslational)	Juvenile HC
Hepcidin (*HAMP*)	19q13.1	Liver, heart, skin and retina	Mature peptide of 25 amino acids	Regulator of iron export to plasma	Diferric TF, BMPs, IL-6, ERFE, TMPRSS6	Juvenile HC
Hephaestin (*HEPH*)	Xq12	Enterocyte basolateral membrane	TMP, copper protein with homology to caeruloplasmin	Fe^{2+} oxidase	Not known	*Sla* mouse: iron deficiency anaemia
IRP1 (*ACO1*)	9p21.1	Widespread	Cytoplasmic	Regulation of FTH, FTL, TFRC, DMT1, ferroportin, ALAS2, HIF-2α	4Fe-4S clusters change it to aconitase	Normochromic anaemia with low ferritin
IRP2 (*IREB2*)	15q25.1	Widespread	Cytoplasmic	As IRP1	Iron-induced proteasomal degradation	Functional iron deficiency Neurodegeneration
Mitoferrin 1 (*SLC25A28*)	8p21	Erythroid, liver, skeletal muscle and heart	Mitochondrial inner Membrane	Mitochondrial iron importer	GATA1 in zebrafish	Not known
Matriptase-2 (*TMPRSS6*)	22q13.1	Mainly liver	Type II transmembrane serine protease	Cleaves haemojuvelin	Hypoxia	*Mask* mouse Autosomal recessive IRIDA
Nuclear receptor coactivator 4 (*NCOA4*)	10q11	Ubiquitous	Iron binding protein	Mediator of ferritinophagy. Regulator of DNA replication origins	Iron-induced proteasomal degradation	Not known

Six-transmembrane epithelial antigen of prostate 3 (*STEAP3*)	2q14.2	Erythroid, placenta (associated with TFR1)	Six-transmembrane epithelial antigen of prostate-3	Ferric reductase (endosomal) also reduces copper	Not known	Human microcytic anaemia
Solute carrier zinc transporter family member 14, ZIP14 (*SLC39A14)*	8p21.3	Widespread	Zinc and other metal transporter	Iron transport receptor for NTBI in liver and pancreas	Not known	Not known
Transferrin (*TF*)	3q21	Liver, all cells	Single-chain polypeptide, glycoprotein	Iron transport. Ligand (diferric) for TFR1 and TFR2	Hypoxia Iron stores (?)	Atransferrinaemia: autosomal recessive anaemia with iron overload
Transferrin receptor 1 (*TFRC*)	3q29	Widespread: highest number in erythroid precursors	TMP, dimeric	Binds transferrin	Fe (3′-IRE)	Embryonic lethality in knock out mouse. Immunodeficiency (*missense* recurrent mutation) in humans
Transferrin receptor 2 (*TFR2*)	7q22	Liver, erythroid precursors, osteoblasts and osteoclasts	TMP dimeric, 60% similarity to TFR1 in extracellular domain	Binds transferrin, regulator of hepcidin synthesis; EPOR partner	No IRE, Stabilised on membrane by diferric transferrin	Human HC, autosomal recessive

* In hyperferritinaemia/cataract syndrome, mutations affect the 5′-UTR IRE. In neuroferritinopathy, mutations affect *FTL* coding sequences.
BMP, bone morphogenetic protein; EPOR, erythropoietin receptor; Fe/S, iron sulfur cluster; HC, haemochromatosis; IL6, interleukin 6; IRE, iron responsive element; IRIDA, iron refractory iron deficiency anaemia; IRP, iron regulatory protein; MDS-RS, myelodysplasia with ring sideroblasts; NTBI, non-transferrin bound iron; TMD, transmembrane domain; TMP, transmembrane protein.

receptors on erythroblasts, which is especially enhanced in expanded erythropoiesis and in iron deficiency (see below).

A single homozygous *TFRC* mutation causes mild anaemia and severe immunodeficiency, strengthening that iron has an essential role in lymphocyte function.

A second receptor, TFR2, also binds diferric transferrin (Table 11.1) although with affinity lower than TFR1. Both receptors bind HFE and through this binding are involved in regulating hepcidin synthesis (see Figure 11.2). TFR2 binds erythropoietin receptor (EPOR) in erythroid precursors, negatively modulating EPO signalling through an unknown mechanism.

Lactoferrin is a glycoprotein that is structurally related to transferrin. It is found in milk and other secretions and in neutrophils. It has a bacteriostatic action at secreting surfaces by depriving microorganisms of the iron needed for their growth.

Divalent metal transporter 1

Divalent metal transporter (DMT)1 is an electrogenic pump that requires proton cotransport in order to transfer Fe^{2+} across cell membranes. This occurs at the apical membrane and subapical endosomes of the duodenal enterocyte and the transferrin-cycle endosome, both of which have a low pH. The intestinal DMT1 is produced by different mRNA splicing that produces endosomal DMT1. DMT1 expression is upregulated in iron deficiency/hypoxia (see later) and may be involved in absorption of other divalent metal cations, including Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+}. The expression of DMT1 and DCYTb is increased in hypoxia by the hypoxia-inducible factor (HIF)-2α.

Ferroportin

This transmembrane domain protein is the basolateral transporter of iron, essential for iron release from macrophages, the intestinal absorptive enterocyte, hepatocytes and placental syncytiotrophoblasts. It is also present in intracellular compartments. Control of ferroportin occurs at multiple levels: inflammatory cytokines reduce ferroportin transcription, while IRPs, interacting with a 5′IRE, control its translation. Caeruloplasmin is required for the cell-surface localisation of ferroportin, whose concentration is post-translationally controlled by hepcidin, which triggers its internalisation and degradation in lysosomes. Hepcidin also blocks the iron channel in the

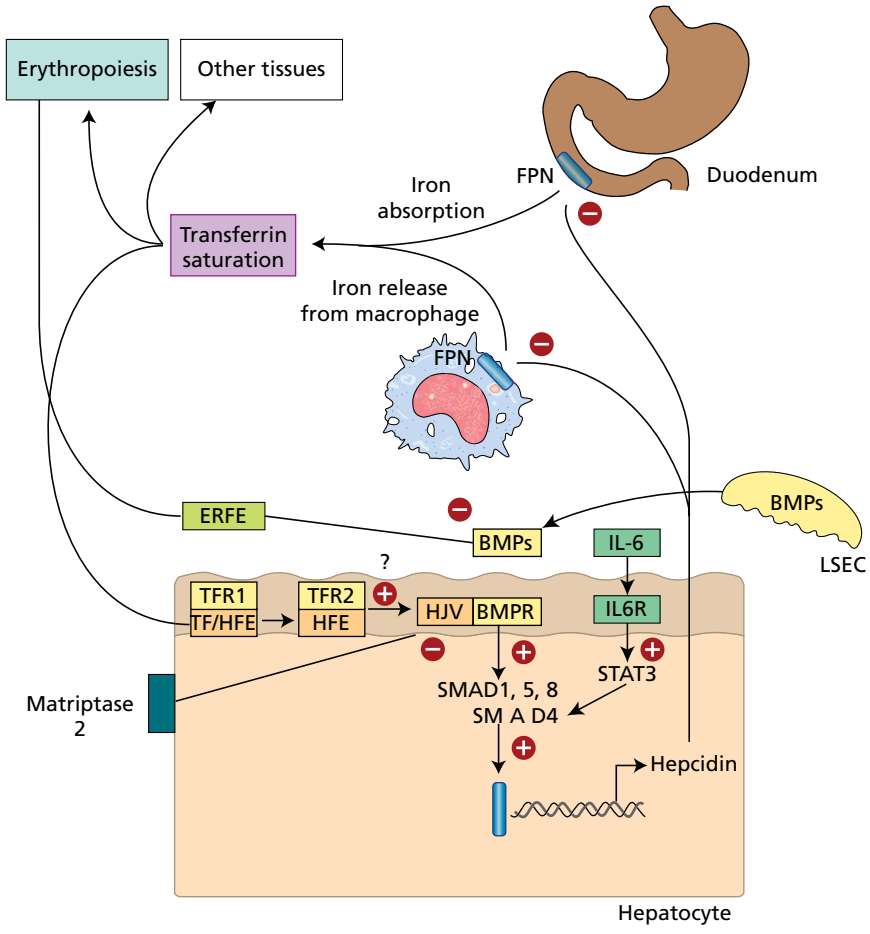


Figure 11.2 Stimulatory and inhibitory signals of hepcidin regulation. Hepcidin, as well as haemojuvelin (HJV), transferrin receptor 2 (TFR2) and HFE, are all produced in the hepatocyte. High plasma iron and inflammation cytokines (as IL-6) stimulate hepcidin synthesis, mediated by SMADs and STAT3, respectively. Conversely, low plasma iron, increased rates of erythropoiesis (including ineffective erythropoiesis) and hypoxia inhibit hepcidin production. This is mediated by matriptase 2 and ERFE. Hepcidin binds ferroportin (FPN), causing its destruction and so inhibits iron absorption and iron release from macrophages into plasma and from intracellular compartments. BMP, bone morphogenetic protein; ERFE, erythroferrone.

central cavity of the iron exported, as shown by the effect of hepcidin in cells unable to internalise ferroportin, as the erythrocytes. How iron homeostasis is finely tuned by the hepcidin-ferroportin axis has been recently explained by cryogenic electro-microscopy structure of the two proteins complex: hepcidin has a strikingly higher (80×) affinity for iron-bound than for iron-free ferroportin, inducing preferential degradation of the first.

Other proteins

The roles in iron metabolism of haemojuvelin (HJV), bone morphogenetic proteins (BMP-6 and BMP2), small mothers against decapentaplegic (SMADs), ferrioxidative and reduction enzymes, and caeruloplasmin are discussed under the headings of hepcidin regulation, iron absorption, iron uptake by erythroid cells and haem synthesis.

Hepcidin

Hepcidin has a central role in the regulation of iron metabolism, absorption and recycling (Figure 11.2). A product of the *HAMP* gene (Table 11.1), it is a small peptide (25 amino acids) released from a large prepropeptide of 84 amino acids. It is predominantly expressed in the liver. It regulates iron homeostasis by blocking the iron export and causing ferroportin internalisation and degradation in lysosomes. It therefore acts to inhibit iron absorption, iron release from macrophages and iron transport across the placenta. It is bound in plasma to α_2-macroglobulin and the major route of clearance is the kidney. Hepcidin can be measured in serum by ELISA or mass spectrometry-based techniques.

Regulation of hepcidin expression

The regulation of hepcidin expression is transcriptional. Hepcidin expression is increased in response to raised serum iron, iron overload and inflammation and is suppressed by iron deficiency, hypoxia and increased erythropoietic activity. Under basal conditions, expression depends on signalling through the BMP/SMAD pathway (Figure 11.2). The ligands BMP6 and BMP2 are cytokines produced by liver sinusoidal endothelial cells. BMP-6 is the master hepcidin activator in response to iron *in vivo* in murine models, while BMP2 likely determines basal hepcidin levels. More recent data suggest that full hepcidin stimulation requires as ligand the formation of BMP2/BMP6 heterodimers complex. HJV is a member of the repulsive guidance molecules (RGM) family that is highly expressed in liver, skeletal muscles and the heart. It is either associated with cell membranes through a glycosylphosphatidylinositol anchor or released as a soluble form. Membrane-bound HJV participates in the pathway regulating hepcidin expression as a BMP coreceptor, whereas soluble HJV is a BMP antagonist in murine models.

HFE and TFR2 are also involved in hepcidin expression (Figure 11.2). HFE is able to bind TFR1 and TFR2. During low or basal serum iron conditions, HFE and TFR1 exist as a complex at the plasma membrane, TFR1 serving to sequester HFE to silence its activity. Diferric serum transferrin (Fe^{2+}-TF) competes with HFE for binding to TFR1. Increased serum transferrin saturation therefore results in dissociation of HFE from TFR1. A model suggests that HFE then binds to TFR2 and conveys the Fe^{2+} TF status to the signal transduction effector complex. HJV binds to BMP interacting with BMP receptors, that phosphorylate SMADs to form a SMAD-1/-5/-8–SMAD-4 complex, which translocates to the nucleus and stimulates hepcidin production by activating its promoter. In keeping with this model, genetic mutations of HFE, TFR2, HJV and hepcidin all result in haemochromatosis with low serum hepcidin levels (see Chapter 12). Liver iron levels also control BMP-6 production by LSEC, but the mechanisms are still unclear.

A second type of transcriptional hepcidin regulation occurs in inflammation. Interleukin (IL)-6 and IL-1β induce transcription of the hepcidin gene by activating STAT3 (signal transducer and activator of transcription 3) that binds to a regulatory element in the hepcidin promoter. The full hepcidin expression in inflammation requires the integrity of the BMP pathway since the two signals (Figure 11.2) converge on a final shared SMAD-4-dependent pathway.

The hepcidin response is remarkably rapid. In humans, iron ingestion results in a sharp increase in serum hepcidin within a few hours of starting treatment lasting up to 48 hours. Likewise, infusion of recombinant IL-6 results in a significant increase in serum hepcidin and decreased serum iron and transferrin saturation within 2 hours of infusion. These observations imply that hepcidin expression is directly controlled by serum iron (through transferrin saturation) and IL-6. These physiological mechanisms explain the onset of anaemia in inflammatory disorders.

Response to anaemia and hypoxia

Hepcidin levels are reduced or undetectable in iron deficiency anaemia. Iron absorption is accelerated in iron deficiency, ineffective erythropoiesis and hypoxia (Figure 11.2), the latter two likely through common mechanisms mediated by the 'erythroid regulator'. In iron deficiency, *TMPRSS6* mediates hepcidin inhibition by attenuating the BMP signalling (see matriptase 2). A direct epigenetic inhibition on the hepcidin locus has also been reported.

Erythroferrone

Erythroferrone (ERFE or FAM123B) is the physiological erythroid regulator in mice. It is released by erythroid precursors and suppresses hepcidin after bleeding and erythropoietin treatment in order to increase intestinal iron absorption and macrophage iron release according to the erythropoietic needs. It is a member of the TNF-alpha-like protein family, that binds and sequesters

several BMPs, in particular BMP6 and likely BMP2/6 heterodimers, reducing their interactions with receptors and the BMP pathway activation. ERFE has a minor, if any, role in iron deficiency while it is essential when erythropoiesis is physiologically expanded by erythropoietin as in hypoxia. Hypoxia directly increases iron absorption through increased duodenal DMT1/DCYTB expression and indirectly through hepcidin suppression by increased erythropoiesis and ERFE. The latter mechanism may contribute to the harmful accumulation of iron in response to chronic anaemia associated with ineffective erythropoiesis in thalassaemia and other dyserythropoietic anaemias.

Matriptase-2

This is a type 2 member of the transmembrane serine protease family encoded by *TMPRSS6* and mainly expressed in the liver. Membrane-bound matriptase-2 regulates hepcidin expression by cleaving membrane-bound HJV or according to other models by cleaving/blocking multiple components of the BMP pathway. The factors that regulate matriptase-2 expression need to be elucidated. Matriptase-2 activity over-rides all known activating stimuli of hepcidin synthesis. Homozygous or compound heterozygous *TMPRSS6* mutations in humans and homozygous inactivation of the gene in mice result in marked upregulation of hepcidin and blockade of intestinal and macrophage iron transport into plasma, leading to a refractory hypochromic microcytic anaemia (see below).

Intracellular iron homeostasis

Synthesis of several of the proteins involved in iron metabolism is regulated at the level of RNA translation by two cytoplasmic iron-dependent proteins, IRP1 and IRP2 (Table 11.1). These are capable of binding to mRNAs that contain a sequence forming a stem-and-loop structure called an *iron-responsive element* or IRE (Figure 11.3). The 3′-untranslated region (3′-UTR) of TFR1 contains five IREs, whereas the 5′-UTR region of ferritin mRNA contains a single IRE. Binding of IRP when there are low levels of intracellular iron protects TFR1 mRNA from cytoplasmic degradation, but inhibits translation of ferritin mRNA, by interfering with the binding of initiation factors. In contrast, when intracellular iron is increased, the opposite effects occur. Thus, coordinated regulation of TFR1 and ferritin acts to maintain a constant intracellular iron content over the short term by balancing cellular iron uptake and storage.

Erythroid δ-aminolevulinic acid synthase (ALAS2) mRNA also has an IRE in its 5′-UTR region, whereas 'housekeeping' ALAS1 mRNA does not. The IRP–IRE system is therefore involved in matching iron supply to haem synthesis, with repression of protoporphyrin synthesis in iron-deficient erythroblasts. An intestinal isoform of DMT1 like TFR1 has a 3′-IRE and is upregulated in iron deficiency. Ferroportin has a 5′-IRE (Table 11.1) but, as occurs for DMT1, ferroportin may be translated in a non-IRE isoform that escapes the IRP control in some tissues, for example in erythroid and duodenal cells. The IRE-IRP system regulates a vast assortment of mRNA. Examples are hypoxia-inducible factor 2 (HIF-2α) and the cell cycle regulator CDC14A. HIF-2α has a 5′-IRE: its translation is repressed in iron deficiency in the kidney, reducing erythropoietin production and erythropoiesis expansion. CDC14A has a single 3′-IRE that acts to stabilize mRNA in condition of iron repletion.

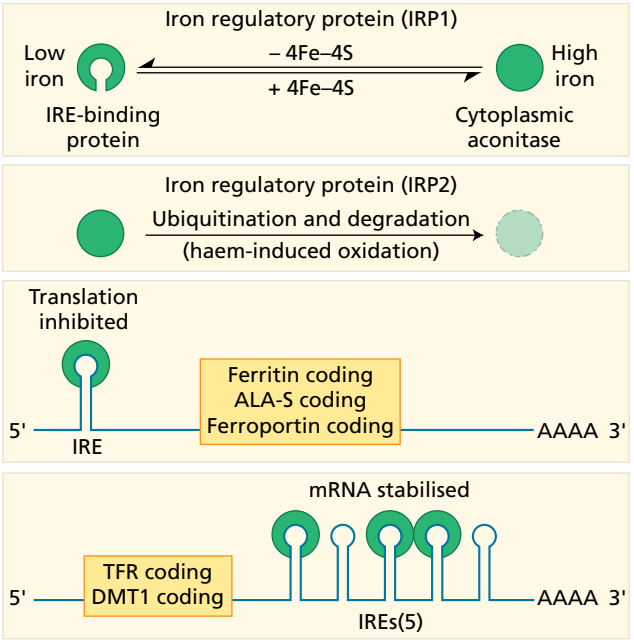


Figure 11.3 Coordinate regulation of expression of ferritin and transferrin receptor: the role of the iron response element (IRE)–iron regulatory protein (IRP) mechanism. When cellular iron levels are low, IRP binds to the IRE stem and loop structures of mRNA to inhibit translation of ferritin, ALA-S2 and ferroportin, but increases translation of transferrin receptors (TFR) and DMT1 by preventing degradation of the mRNA. When iron levels are high, the IRP functions as a cytoplasmic aconitase and no longer binds to the IREs. Ferritin, ALAS2 and ferroportin synthesis can thus proceed, while TFR and DMT1 synthesis is reduced. IRP2 binds to IREs when iron levels are low, but is degraded after ubiquitination (initiated by haem-induced oxidation) when iron levels are high. The IRPs therefore provide two ways of sensing iron requirements, either involving Fe–S proteins or haem proteins.

Normal iron balance

The amount of iron in the body at birth depends on the blood volume and haemoglobin concentration, birth weight (which determines blood volume) being particularly important. Delay in clamping the cord leads to an increased red cell mass by placental transfusion. The level of maternal iron stores has little effect on

Table 11.2 Daily iron losses and requirements.

Group (age, years)	Daily loss Urine, skin, faeces, etc.	Menses (mg)	Requirement for growth (mg)	Total (mg)
Children				
0.5–1	0.17	—	0.55	0.72
1–3	0.19	—	0.27	0.46
4–6	0.27	—	0.23	0.50
7–10	0.39	—	0.32	0.71
Males				
11–14	0.62		0.55	1.17
15–17	0.90	—	0.60	1.50
18+	1.05	—	—	1.05
Females				
11–14*	0.65	—	0.55	1.20
11–14	0.65	0.48†	0.55	1.68
15–17	0.79	0.48†	0.35	1.62
18+	0.87	0.48†	—	1.35
Post menopause	0.87	—	—	0.87
Pregnancy 2nd trimester	0.70	—	2.1‡	2.8
Pregnancy 3rd trimester	0.70	—	3.3‡	4.0
Lactating	1.15	—	—	1.15

* Non-menstruating
† Median loss
‡ Placental and fetal growth
(*Source: Adapted from* WHO (2001) *Iron Deficiency Anaemia. Assessment, Prevention and Control. A Guide for Programme Managers*. World Health Organization, Geneva.)

fetal iron. The newborn contains about 80 mg/kg at full term. Neonatal iron reserves are utilised for growth and from 6 months to 2 years virtually no iron stores are present. Thereafter, iron stores gradually accumulate during childhood to around 5 mg/kg. In men, there is a further increase between 15 and 30 years to about 10–12 mg/kg (total up to approximately 1 g), whereas iron stores remain lower in women (average 300 mg) until the menopause.

Requirements are higher in menstruating women and during periods of rapid growth in infancy and adolescence (Table 11.2). Menstrual blood loss has a median value of 30 mL, but the 95th centile value is 118 mL per month (equivalent to 1.9 mg iron per day), which has been found to be significantly associated with iron deficiency. Requirements are highest of all in pregnancy, about 3 mg in the second and about 4 mg in the third trimester.

Iron absorption

Iron absorption depends not only on the amount of iron in the diet but also, and more importantly, on the bioavailability of that iron, as well as the body's needs for iron. A normal Western diet provides approximately 15 mg of iron daily. Of that iron, digestion within the gut lumen releases about half in a soluble form, from which about 3 mg may be taken up by mucosal cells and only about 1 mg (or 5–10% of dietary iron) transferred to the portal blood in a healthy man. Iron absorption can thus be influenced at several different stages.

Dietary and luminal factors

Much of dietary iron is non-haem iron derived from cereals (commonly fortified with additional iron in the UK), with a lesser component of haem iron from meat and fish. Even in iron deficiency, the maximum iron absorption from a mixed Western diet is no more than 3–4 mg daily. This figure is much less with the predominantly vegetarian, cereal-based diets of most of the world's population since iron is better absorbed from animal than vegetable sources.

Iron is released from protein complexes by acid and proteolytic enzymes in the stomach and small intestine, and haem is liberated from haemoglobin and myoglobin. Iron is maximally absorbed from the duodenum and less well from

the jejunum, probably because the increasingly alkaline environment leads to the formation of insoluble ferric hydroxide complexes. Acid pH, vitamin C and some low-molecular-weight chelates (e.g. sugars and amino acids) enhance absorption. Therapeutic ferrous iron salts are well absorbed on an empty stomach, but when taken with a meal, absorption is reduced as a result of the same ligand binding processes that affect dietary non-haem iron; phytates, tannates in tea and bran inhibit absorption.

Mucosal factors: molecular aspects of iron absorption and its regulation

A variety of mechanisms for the binding of non-haem iron to the mucosal membrane have been described. Specific, saturable and receptor-mediated mechanisms, and passive diffusion at higher doses, may occur. Iron absorption is regulated both at the stage of mucosal uptake and at the stage of transfer to the blood. The process is illustrated in Figure 11.4. Non-haem iron is released from food as Fe^{3+} and reduced by duodenal cytochrome b_1 (DCytb) to Fe^{2+}. This is transported across the brush border membrane by DMT1, which is upregulated in iron deficiency and by HIF-2α activation in the local hypoxic environment of duodenum. It is assumed that iron enters the labile pool and some may be incorporated into ferritin and lost when the cells are exfoliated. Iron destined for retention by the body is transported across the basolateral membrane by ferroportin before uptake by transferrin as Fe^{3+}. The regulation of duodenal cell iron release by hepcidin through its action on ferroportin is rapid. Hephaestin is a copper-containing ferroxidase expressed predominantly in villous cells of the small intestine that converts Fe^{2+} to Fe^{3+} in the basolateral transfer step of iron absorption.

Haem iron initially bound to an unknown receptor at the brush border membrane is released intracellularly by haem oxygenase before entering the labile iron pool and following a common pathway with iron of non-haem origin.

Microbiota in the gut compete for iron, producing metabolites (as 1,3-diaminopropane and reuterin) that inhibit HIF-2α function in iron deficiency, decreasing iron absorption in mice. However, data in humans are still lacking.

Iron uptake by erythroid cells

About 85% of transferrin iron normally enters developing red cells for incorporation into haemoglobin. This tissue distribution of transferrin-bound iron reflects the expression of transferrin receptors, which are present in high concentration of cells with a high iron requirement. The latter includes any rapidly dividing cells but is normally dominated by the cells of the erythron. A soluble truncated form of the transferrin receptor derived from these cell surfaces is detectable in serum.

Transferrin receptors have the highest affinity for diferric transferrin. The transferrin–receptor complex is taken up by a process of receptor-mediated endocytosis (Figure 11.5). The iron is released at the low pH of the endosome, reduced from Fe^{3+} to Fe^{2+} by STEAP3, a ferrireductase, before the apotransferrin and receptor are recycled to the plasma and the cell membrane, respectively. Iron release from the endosome is via DMT1 (Figure 11.5) and the iron is transported into mitochondria by mitoferrin or enters ferritin. A mitochondrial version of ferritin, which likely protects mitochondria from iron-induced oxidative damage, is elevated in congenital or acquired sideroblastic anaemias. Direct transfer of ferritin from macrophages to erythroblasts (rhopheocytosis) in the erythroblastic islands of the bone marrow may also occur.

Some 80–90% of iron taken into developing erythroblasts is converted to haem within 1 hour. Any iron taken up in excess of the requirement for haem synthesis is incorporated in ferritin (Figure 11.5). Mitochondrial iron is also utilised for the production of iron/sulfur clusters, prosthetic groups essential for enzymes of the haem synthesis in erythroid cells, as ferrochelatase and aminolevulinic acid dehydratase. This important role is clearly

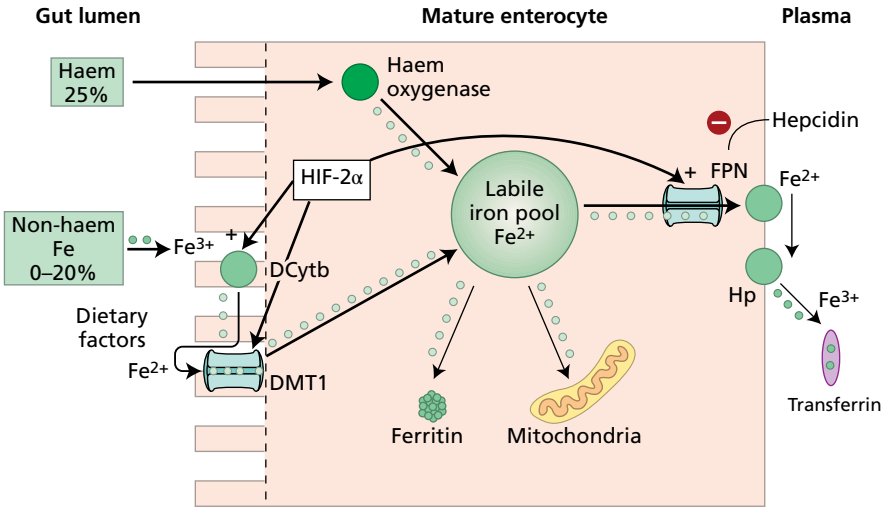


Figure 11.4 Molecular pathways of iron absorption HIF-2α and hepcidin dependent. The area enclosed in the dotted box refers to the uptake of iron from the plasma in the developing enterocyte in the intestinal crypt. Otherwise, the diagram refers to iron absorption by the villous epithelial cell. DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; FPN, ferroportin; Hp, hephaestin. For further details see text and Table 11.1.

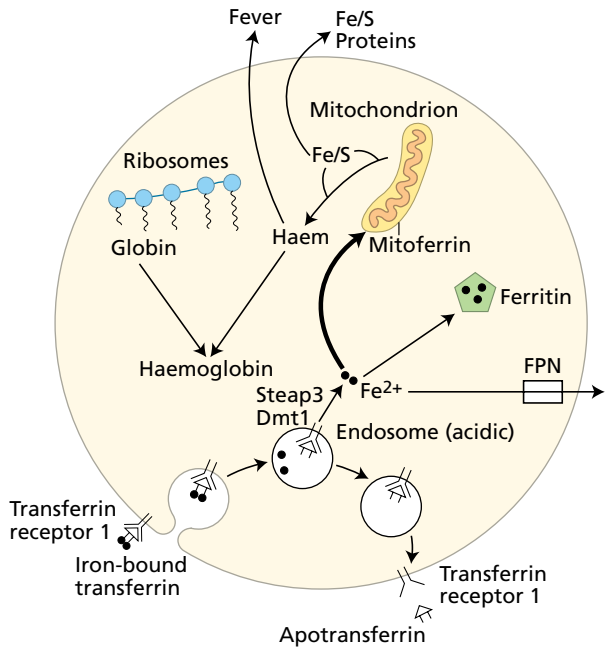


Figure 11.5 Incorporation of iron from plasma transferrin into haemoglobin in developing red cells. Uptake of transferrin iron is by receptor-mediated endocytosis. Endosomal DMT1, divalent metal transporter 1; STEAP3: endosomal metalloreductase; FLVCR, feline leukaemia virus C receptor. FPN, ferroportin.

strengthened by rare forms of sideroblastic anaemia due to mutations of proteins involved in biogenesis of iron–sulfur cluster (see below). The red cell ferritin content is therefore increased when haemoglobin synthesis is impaired, as in thalassaemia where excess iron may be seen in the cytoplasm of mature red cells as one or more siderotic granules. These are composed of haemosiderin, and stain blue with Perls' reaction and purplish blue with Romanowsky stains, when they are called Pappenheimer bodies. The spleen removes these granules by its pitting action. In sideroblastic anaemia, iron is trapped in mitochondria giving origin to ringed sideroblasts (see below) while the cytosol is relatively iron-poor.

Haem synthesis and mitochondrial iron metabolism

Haem consists of a protoporphyrin ring with an iron atom at its centre. Haem is synthesised from the precursors succinyl-CoA and glycine, which condense to form δ-aminolevulinic acid (ALA) under the action of ALAS, with pyridoxal phosphate as a coenzyme. Housekeeping ALAS is ALAS1; in erythroid cells, erythroid-specific ALAS2 predominates. ALA can be utilised for the formation of both purines and haem. Four molecules of porphobilinogen condense under the influence of porphobilinogen deaminase and uroporphyrinogen cosynthase to form the tetrapyrrole ring compound uroporphyrinogen III. The latter is converted to protoporphyrin IX. Finally, iron in the ferrous form is incorporated by the enzyme ferrochelatase. Iron in haem has six coordinating valencies: four links the iron to nitrogen atoms in each pyrrole ring, whereas the remaining two link haem to histidine residues in the globin chain, the distal bond being unstable and easily replaced by oxygen to form oxyhaemoglobin. A haem exporter, group C feline leukaemia virus receptor (FLVCR) (Table 11.1) (Figure 11.5), is present in erythroid cells and rids them of any toxic haem made in excess.

The mitochondria play a major role in haem synthesis as they contain ALAS, coproporphyrinogen oxidase and ferrochelatase, the enzyme sequence from ALA to coproporphyrinogen being situated in the cytoplasm. Besides haem, mitochondria utilise iron in the synthesis of iron–sulfur clusters, prosthetic groups essential for the function of several mitochondrial (respiratory chain complexes, ferrochelatase, aconitase), cytosolic (δ-aminolevulinic acid dehydratase, aconitase) and nuclear proteins (DNA helicase, DNA repair enzymes). A number of porphyrins are formed by side reactions during the synthesis of protoporphyrin. In the porphyrias (see below), many of these compounds accumulate in the major sites of haem synthesis, the liver and the red cells.

The assembly of iron–sulfur clusters is a complex process that requires multiple proteins. The best known is frataxin, a likely iron donor in this pathway. The gene encoding frataxin is mutated in Friedreich ataxia. *ABCB7*, a member of a family of transmembrane proteins characterised by the ABC domain that binds and hydrolyses ATP, transfers iron–sulfur clusters from mitochondria to the cytosol and interacts with other ABC members and ferrochelatase in the haem synthetic pathway. Mutations of *ABCB7* cause an X-linked form of sideroblastic anaemia with ataxia (see below). Other proteins active in the pathway, as glutaredoxin-5 (GLRX5), heat shock protein (HSP)A9 and heat shock cognate protein 20 (HSC20 or HSCB), that facilitate the transfer of nascent 2-iron, 2-sulfur clusters to recipient proteins have been found mutated in rare recessive forms of sideroblastic anaemia. Iron–sulfur cluster convert IRP1 to aconitase to stimulate the citric acid cycle; at the same time, this precludes the IRE binding activity and signals iron sufficiency.

The mitochondria are also the site of the citric acid cycle, which supplies succinate to initiate haem synthesis. The mature red cell, which lacks mitochondria, is therefore unable to synthesise haem. Overall these data confirm the essential role of iron–sulfur clusters for erythropoiesis, highlight the coordination of iron–sulfur clusters and haem synthesis and cell metabolism and the requirement for haem synthesis of an intact Fe/S cluster biogenesis.

Intracellular transit iron and plasma non-transferrin-bound iron

It has been suggested, but not experimentally proved, that there is a transit pool of 'metabolically active' or 'labile' iron within cells, which receives iron from degraded haem or ferritin, exchanges with transferrin and is incorporated into newly synthesised

iron-containing proteins. This iron is considered to be sensed by iron regulatory proteins and available for chelation. Within cells, low-molecular-weight chelates (e.g. citrate) may be present. In iron overload, non-transferrin-bound iron (NTBI) may also exist as oligomeric iron oxide, when transferrin saturation exceeds 50–60%, either free or bound to albumin, and is particularly toxic to organs such as liver and pancreas that uptake NTBI through the cation (Zinc) transporter ZIP14 (*SLC39A14*) (Chapter 12).

Breakdown of haemoglobin and iron recycling

After phagocytosis by macrophages, haem from senescent red cells is broken down by haem oxygenase (HMOX1) to release iron. As ferrous iron, it can then either enter ferritin (where it is oxidised to ferric iron by the ferritin protein) or be released into plasma (via ferroportin), where its binding to transferrin (also as the ferric form) may be facilitated by a plasma ferrous oxidase (e.g. caeruloplasmin). The release of macrophage iron provides most (25 mg/day) of iron for erythropoiesis and is controlled by hepcidin, with high levels, as in inflammation or iron overload, reducing iron release and even causing anaemia. Changes in hepcidin concentration may account for the diurnal rhythm of serum iron concentration, which is highest in the morning and lowest in the evening. A diurnal increase in serum hepcidin at noon and 8 p.m. is observed in healthy volunteers.

Diagnostic methods for investigating iron metabolism

The large amount of iron present as haemoglobin means that the degree of any anaemia must always be considered in assessing iron status. Reduced amounts of haemoglobin accompany an overall reduction in body iron in iron deficiency anaemia or after acute blood loss. In other anaemias, including the anaemia of inflammation and most haemolytic and megaloblastic anaemias, iron is redistributed from the red cells to macrophage iron stores, with a corresponding increase in marrow-stainable iron and serum ferritin. No single measurement is ideal for all clinical circumstances, as all are affected by confounding factors and changes may develop sequentially (as in progressive negative iron balance) or may affect particular body iron compartments. Table 11.3 summarises the changes in measures of iron status accompanying various types of hypochromic anaemia. The assessment of *iron overload* is discussed in Chapter 12.

Serum ferritin

In healthy subjects, the serum ferritin concentration correlates with iron stores. Normal concentrations of serum ferritin range from about 30–300 μg/L and are higher in men (median about 90 μg/L) than in premenopausal women (median 30 μg/L). In neonates, the concentration in cord blood (median approximately 100 μg/L) rises further over the first 2 months of life as fetal haemoglobin is broken down, and thereafter falls to low levels (median 20–30 μg/L) throughout childhood and adolescence.

Serum ferritin concentrations below 15 μg/L are virtually specific for storage iron depletion, but normal values do not exclude this. Values <30 μg/L are accepted as more specific and sensitive cut-off for iron deficiency. On the contrary values above 300 μg/L do not necessarily, or even usually, indicate iron overload. This is because ferritin synthesis is influenced by factors other than iron (in particular, it behaves as an acute-phase reactant in many inflammatory diseases). For this reason, serum ferritin concentrations <100 μg/L may be associated with a lack of storage iron in patients with the anaemia of

Table 11.3 Differential diagnosis of hypochromic anaemia.

	Iron deficiency	Inflammation	Thalassaemia trait (α or β)	Sideroblastic anaemia	IRIDA
MCV/MCH	↓	N or ↓	↓	↓ (congenital) ↑ or N (acquired)	↓
Serum iron	↓	↓	N	↑	↓
TIBC	↑	↓ or N	N	N	↑
Transferrin saturation	↓	↓	N	↑	↓
Serum ferritin	↓	N or ↑	N	↑	N
Serum TFR	↑	N	N	N or ↑	↑
Serum hepcidin	↓	↑	N or mildly ↓	↓	N or ↑
Bone marrow iron stores	↓	N or ↑	N	N or ↑	N or ↑
Erythroblast iron	↓	↓	N	Ring forms	

IRIDA, iron refractory iron deficiency anaemia; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; N, normal; TFR, transferrin receptor; TIBC, total iron-binding capacity.

inflammation, or anaemia of chronic disease. When serum ferritin is an unreliable indicator of iron deficiency because of concomitant inflammation low transferrin saturation is used as marker of deficiency (see below).

Bone marrow aspiration

Staining the bone marrow for iron gives an indication of stores as well as erythroblast iron (Figure 11.6a). In iron deficiency anaemia, macrophage and erythroblast iron are absent (Figure 11.6b). In the anaemia of chronic diseases/inflammation macrophage iron is present, but erythroblast iron is absent.

Serum iron and iron-binding capacity

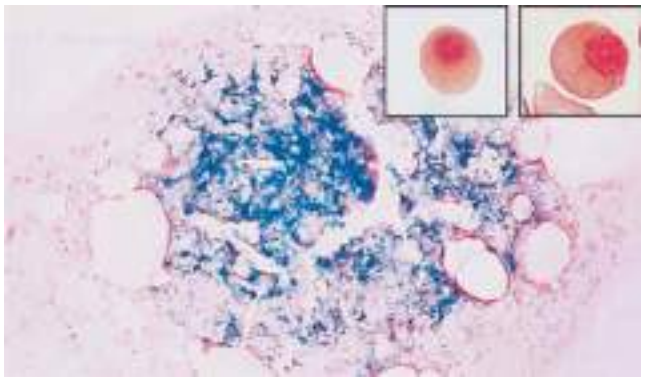
The serum iron and, more particularly, the saturation of the total iron-binding capacity of transferrin (TIBC) gives a measure of the iron supply to the tissues. A serum transferrin saturation of less than 15% is insufficient to support normal erythropoiesis. A rise in TIBC is characteristic of iron deficiency. A reduced serum iron concentration with a normal or reduced TIBC is a characteristic response to inflammation (see below). A value <20% is considered an indicator of iron deficiency in inflammation.

Serum transferrin receptors

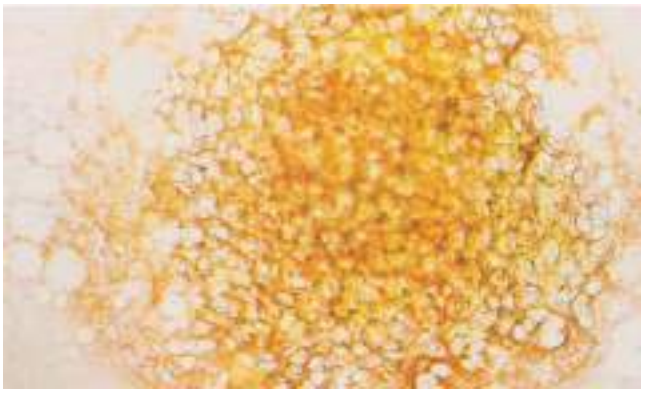
Plasma concentrations of soluble transferrin receptors (TFRs) reflect both the number of erythroid precursors and iron supply to the bone marrow, TFRs being raised in erythroid hyperplasia and in iron deficiency. In anaemia of inflammation, the assay has been proposed as a valuable indicator of deficiency of body iron stores. Serum TFRs only increase in this situation in the absence of storage iron. The test, however, is not standardised amongst different laboratories and that limits its clinical use.

Red cell protoporphyrin

When iron supply to the erythron is limited, iron incorporation into haem is restricted, leading to accumulation of the immediate precursor, protoporphyrin IX bound to zinc. This is lost only slowly from circulating red cells; concentrations greater than the normal upper limit of 80 µmol/mol haemoglobin, therefore, indicate that a reduction in iron supply has been present over the previous few weeks. Protoporphyrin levels may also increase in patients with sideroblastic anaemias, except those due to *ALAS2* mutations, and in lead poisoning. The test is not uniformly available, but analysers that are portable and easy to operate are useful in large-scale field studies of iron deficiency anaemia as an initial screening test.



(a)



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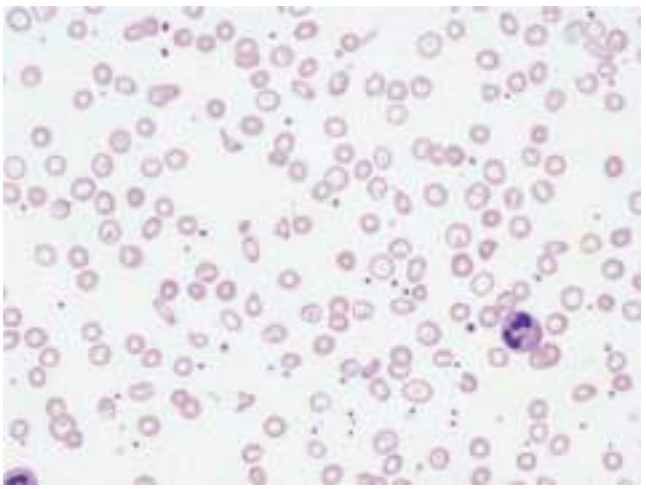

(c)

Figure 11.6 (a) A bone marrow showing plentiful iron in macrophages (Perls' stain) with iron granules in erythroblasts (insets). (b) Iron deficiency: bone marrow showing absence of stainable iron (Perls' stain). (c) Iron deficiency: peripheral blood film showing hypochromic microcytic red cells.

Percentage of hypochromic red cells

As iron supply to the erythron diminishes, the new red cells produced are increasingly hypochromic. Assessment of the

haemoglobin content of individual red cells, which is possible using some automated cell counters, allows measurement of the percentage of hypochromic cells. Values rising to above 6% may help in the early identification of impaired iron supply in patients with chronic kidney injury who are receiving treatment with recombinant erythropoietin, when associated inflammatory disease means that other measures of iron status can be misleading. The utility of this assay in other anaemias of inflammation to diagnose a concomitant iron deficiency is under evaluation.

Reticulocyte haemoglobin content

Measurement of the reticulocyte content of haemoglobin is now used as a test of functional iron status. This parameter (CHr or Re–He according to the type of automated counter used) is useful in screening iron status, particularly for the assessment of iron-deficient erythropoiesis, as in dialysis patients treated with erythropoietin. Moreover, CHr may serve as a predictor of the response of anaemia to iron treatment. When response to treatment is positive, an increase in CHr may be discerned within a few days of starting treatment before overall mean corpuscular haemoglobin (MCH) has changed.

Iron deficiency

Iron deficiency may be absolute or functional. Absolute iron deficiency entails a decrease in total body iron including circulating, tissue and stored iron. Functional iron deficiency refers to conditions where circulating iron is low, tissue iron may be decreased, while iron stores are normal or even increased; it is a form of iron sequestration and tissue maldistribution, prevalently related to inflammatory conditions. Functional iron deficiency may occur also in patients treated with erythropoiesis-stimulating agents, where the demand for iron by the expanded erythron may outstrip the ability to mobilise iron from stores. When used without specification iron deficiency refers to absolute iron deficiency.

Absolute iron deficiency

Usually, iron deficiency develops slowly and anaemia is the final outcome of a sequence of events that start with depletion of iron stores, followed by iron-deficient erythropoiesis and culminate with decreased red blood cell production.

When the body is in a state of negative iron balance, the first event is depletion of body stores, which are mobilised for haemoglobin production. Iron absorption is increased when stores are reduced, before anaemia develops and even when the serum iron level is still normal, although the serum ferritin will have already fallen.

With further iron depletion, manifested by a serum ferritin below 15 μg/L and a fall in serum transferrin saturation to less than 15%, iron-deficient erythropoiesis develops with increasing concentrations of serum transferrin receptor and red cell protoporphyrin. At this stage, the haemoglobin, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) may still be within the reference range, although they may rise significantly when iron therapy is given.

If there is no progression iron deficiency is present without anaemia. Even in these cases, symptoms may occur due to lack of iron for proteins and enzymes in different tissues.

Iron deficiency anaemia

If the negative balance continues, frank iron deficiency anaemia develops. The red cells become obviously microcytic and hypochromic (Figure 11.6c) and poikilocytosis becomes more marked. The MCV and MCH are reduced, and target cells may be present. The reticulocyte count is low for the degree of anaemia. The serum TIBC rises and the serum iron falls, so that the percentage saturation of TIBC is usually less than 10%.

The erythroblasts, devoid of siderotic granules, have a ragged vacuolated cytoplasm and relatively pyknotic nuclei. The bone marrow macrophages show a total absence of iron, except where very rapid blood loss outstrips the ability to mobilise the storage iron. Platelets are frequently increased.

The evaluation of the global anaemia burden in the last 20 years showed that, notwithstanding some recent improvement, iron deficiency remains the top-ranking cause of anaemia worldwide, especially in developing countries, due to a combination of inadequate iron intake and blood loss from hookworm, *Schistosomiasis* infestation and other causes of haemorrhage.

Functional iron deficiency

This condition develops when there is a disproportion between high iron demand and limited supply. Examples are anaemia of inflammation in which iron supply becomes limited and erythropoietin administration when stimulates excessive erythropoiesis.

Anaemia of inflammation

Anaemia of inflammation is the second most frequent anaemia worldwide and the first amongst hospitalised patients. It affects patients with underlying chronic disorders characterised by immune activation including chronic infections, autoimmune disorders, cancer.

The sequence of events in anaemia of inflammation starts with the recruitment of inflammatory cells and the production of cytokines as IL-1beta, IL-6, TNF-alpha and INF-gamma. These molecules reduce erythropoietin production by the kidney and bone marrow response to erythropoietin, increasing myeloid at the expenses of erythroid commitment. IL-6 also stimulates hepcidin transcription, causing a rapid reduction of iron absorption and recycling and iron-restricted erythropoiesis. High hepcidin impairs serum iron and transferrin saturation and causes anaemia in the presence of high ferritin. Decreased

red cell survival is a component of anaemia in acute inflammation associated with macrophage activation.

Although iron deficiency anaemia and anaemia of inflammation are distinct entities, they may frequently coexist in a sort of mixed anaemia, often difficult to diagnose.

Tissue effects of iron deficiency

When iron deficiency is severe and chronic, widespread tissue changes may be present, including koilonychia (ridged or spooned nails, breaking easily), hair thinning, angular stomatitis, glossitis and pharyngeal webs (Paterson–Kelly syndrome). Partial villous atrophy, with minor degrees of malabsorption of xylose and fat, reversible by iron therapy, has been described in infants suffering from iron deficiency, but not in adults. Pica is sometimes present; in some who eat clay or chalk, this may be the cause rather than the result of iron deficiency.

Iron-dependent enzymes in the tissues are usually better preserved than other iron-containing compounds. In severe iron deficiency, however, these enzymes are not inviolate and their levels may fall. This may be partly responsible for the general tissue changes, poor lymphocyte transformation and diminished cell-mediated immunity, and impaired intracellular killing of bacteria by neutrophils.

A particular concern has been the finding that infants with iron deficiency anaemia may have impaired mental development and function and that this deficit may not be completely restored by iron therapy. It remains controversial whether impaired work performance seen in adults results from the anaemia or from depletion of mitochondrial iron-containing enzymes.

Tissue iron deficiency may be present in anaemia of inflammation, due to the reduction of circulating iron. Tissue effects of iron deficiency can occur even in the absence of anaemia as exemplified by chronic heart failure where correction of the deficiency by intravenous iron improves symptoms even in the absence of anaemia (see below). Treatment with intravenous iron in non-anaemic premenopausal women with fatigue and serum ferritin <15 µg/L, significantly improved fatigue compared to a placebo-treated group; however, the same treatment seems not to benefit asymptomatic patients. Thus, in the absence of anaemia, it is suggested to treat symptomatic iron deficiency while carefully monitoring asymptomatic patients. In all cases, a cause should be sought.

Causes of absolute iron deficiency

The main causes of absolute iron deficiency are summarised in Table 11.4.

Table 11.4 Causes of absolute iron deficiency.

Dietary
Especially vegetarian diet
Malabsorption
Gluten-induced enteropathy (child or adult), gastrectomy, duodenal bypass, atrophic gastritis, IBD, clay eating, etc.
Blood loss
Uterine: menorrhagia, postmenopausal bleeding, parturition
Gastrointestinal: oesophageal varices, hiatus hernia, *Helicobacter pylori*, peptic ulcer, aspirin ingestion, hookworm, schistosomiasis, hereditary telangiectasia, carcinoma of the stomach, caecum or colon, ulcerative colitis, angiodysplasia, Meckel diverticulum, diverticulosis, haemorrhoids, etc.
Renal tract: haematuria (e.g. renal or bladder lesion), haemoglobinuria (e.g. paroxysmal nocturnal haemoglobinuria, mechanical haemolyis)
Pulmonary tract: overt haemoptysis, idiopathic pulmonary haemosiderosis
Widespread bleeding disorders
Frequent blood donors
Self-inflicted (Munchausen syndrome)
Genetic
Iron refractory iron deficiency anaemia (IRIDA)

Diet

Defective intake of iron is rarely the sole or major cause of iron deficiency in adults in Western communities. It takes more than 4 years for an adult male to develop iron deficiency anaemia on a diet completely devoid of iron. The diet may contain insufficient or poorly available iron as a result of poverty, religious tenets or food faddism. Iron deficiency is more likely to develop in subjects taking a largely vegetarian diet – the majority of the world's population – and who also have increased physiological demands for iron.

Increased physiological iron requirements

Iron deficiency is common in infancy, when demands for growth may be greater than dietary supplies. It is aggravated by prematurity, infections and delay in mixed feeding. Young children (<5 years) in low and middle-income areas are the age group most affected, with little variation in prevalence of anaemia over the last 20 years, according to a recent large study. Iron deficiency is also frequent in adolescence and in females (Table 11.2). The gender gap in iron deficiency anaemia has widened in recent years on a worldwide basis. Pregnancy also favours iron deficiency. The fetus acquires about 280 mg of iron and a further 400–500 mg is required for the temporary expansion of maternal red cell mass. Another 200 mg of iron is lost with the placenta and with bleeding at delivery. Although iron absorption increases throughout pregnancy and increased requirements are partly offset by amenorrhoea, this may not be sufficient to meet the resultant net maternal outlay of over 600 mg iron. Routine iron prophylaxis is not recommended in pregnancy in the UK, but is given for

anaemia (haemoglobin <110 g/L in first trimester, <105 g/L in second or third trimesters and <100 g/L postpartum).

Blood loss

Blood loss is the most common cause of iron deficiency in adults. A loss of more than about 6–8 mL of blood (3–4 mg iron) daily becomes of importance, as this equals the maximum amount of iron that can be absorbed from a normal diet. The loss is usually from the genital tract in women or from the gastrointestinal tract in either sex. The most common cause on a worldwide basis is infestation with hookworm, in which anaemia is related to the degree of infestation. Another important cause in developing countries that can affect any age is schistosomiasis, infection by *Schistosoma (S.) mansoni* causing bleeding from the gut and *S. haematobium* from the bladder, although in both cases anaemia of inflammation may coexist with iron deficiency. In the UK, menorrhagia, haemorrhoids and peptic ulceration are common, as well as gastric bleeding because of salicylates or other non-steroidal anti-inflammatory drugs, hiatus hernia, colonic diverticulosis and cancer (Table 11.4). Haemodialysis results in loss of 4–8 mg iron daily. Investigation often involves, particularly in older subjects, occult blood tests, upper and lower gastrointestinal endoscopy or computed tomography pneumocolon and if these are negative capsule endoscopy. Serum tumour markers CEA and Ca19-9, helpful in monitoring colon cancer when increased, are less useful for screening since normal values do not exclude the possibility of colon cancer being present.

Some unusual causes of blood loss also deserve mention. Cow's milk intolerance in infants may lead to gastrointestinal haemorrhage. Self-induced haemorrhage may occur as an unusual form of Munchausen syndrome. Chronic intravascular haemolysis, such as that in paroxysmal nocturnal haemoglobinuria or mechanical haemolytic anaemia, may be a serious source of urinary iron loss.

Malabsorption

Malabsorption may be the primary cause of iron deficiency, or it may prevent the body from adjusting to iron deficiency from other causes. Dietary iron is poorly absorbed in gluten-induced enteropathy, in both children and adults. Gluten-induced enteropathy is encountered in about 5% of patients presenting with unexplained iron deficiency anaemia and, conversely, about 50% of patients with newly diagnosed coeliac disease have coexistent iron deficiency anaemia. Patients with this disease often show decreased or no response to oral therapy with inorganic iron.

Helicobacter pylori gastritis appears to be a common cause of iron deficiency, responding favourably to eradication with triple therapy. *H. pylori* gastritis inhibits gastric hydrochloric acid secretion, interfering with the solubilisation and absorption of inorganic food iron, but it is also possible that gastrointestinal blood loss plays a significant role in the causation of the iron deficiency. Achlorhydria associated with autoimmune gastritis, an entity that may progress to pernicious anaemia, is an important cause of iron malabsorption due to impaired food iron solubilisation. It is encountered in about 20% of patients with unexplained or refractory iron deficiency anaemia, mostly women of fertile age in whom achlorhydria aggravates the consequences of menstrual blood loss. In inflammatory bowel diseases iron deficiency may coexist with anaemia of inflammation.

Genetic iron deficiency

Homozygous or doubly heterozygous mutations of matriptase-2 are a cause of iron refractory iron deficiency anaemia (IRIDA). The mutations may affect different conserved domains of the protein, including the trypsin-like serine protease domain. The patients show a microcytic hypochromic anaemia due to inappropriately high hepcidin levels and low serum iron and percentage saturation of iron-binding capacity. The patients absorb iron poorly and are refractory to oral iron therapy but are partially responsive to parenteral iron. In IRIDA serum hepcidin (high) is discrepant with transferrin saturation (low); the TSAT/hepcidin ratio is a proposed tool to help with diagnosis.

Iron deficiency in clinics is often multifactorial. Examples are reduced intake of bioavailable iron and blood loss as in low-income countries or vegetarians and vegan or malabsorption and blood loss.

Causes of anaemia of inflammation

Anaemia of inflammation is mainly associated with chronic infectious or autoimmune diseases, advanced cancer, chronic kidney disease (CKD), congestive heart failure and a proportion of cases of anaemia in the elderly. The cytokine effect is common to all types, while some features may characterise specific forms, as erythropoietin deficiency in CKD or cardiomyocyte iron deficiency in congestive heart failure.

Anaemia of inflammation is more and more recognised in disorders with subtle, even subclinical inflammation as obesity, diabetes and other metabolic conditions, as outlined in Table 11.5.

Features of the anaemia of inflammation

The anaemia of inflammation is heterogeneous and multifactorial according to the underlying disorder. Common aspects are a moderate degree of anaemia, (Hb around 80–100 g/L), hypoferraemia, and hyperferritinaemia. Red cells are normocytic and normochromic, unless the degree of anaemia is severe; in these cases, anaemia is microcytic often associated with true iron deficiency. In patients with acute, severe inflammation evidence of haemolysis may be present.

Table 11.5 Causes of functional iron deficiency, anaemia of inflammation or mixed anaemia.

Condition	Details
Infections	Bacterial, viral (including COVID-19), parasitic, fungal
Autoimmune disorders	Rheumatoid arthritis, SLE, autoimmune vasculitis, etc.
Inflammatory bowel diseases	During flare-ups
Chronic kidney disease (CKD)	End-stage dialysis patients
Advanced cancer	GI tract cancer, lymphoma, myeloma
Critically ill patients	Intensive Care Unit patients
Chronic heart failure	Subclinical inflammation
Chronic obstructive pulmonary disease	Especially during exacerbations
Obesity	Subclinical inflammation
Anaemia of the elderly	Part (1/3) of cases
ESA treatment	CKD, MDS, cancer

CKD, chronic kidney disease; ESA, erythropoiesis-stimulating agents; GI, gastrointestinal; MDS, myelodysplastic syndrome; SLE, systemic lupus erythematosus.

The iron restriction induced by cytokines may worsen in chronic disorders for several reasons such as anorexia, malnutrition, decreased absorptive mucosa, blood loss, side effects of drugs such as antithrombotic or proton pump inhibitors, age and comorbidities. Examples of mixed anaemia are the already mentioned *Schistosoma* infections in low-income countries and inflammatory bowel disease, whose components are inflammation and blood loss, especially during acute flares. Iron deficiency in congestive heart failure is due to malabsorption and blood loss often due to drug side effects. CKD patients under dialysis have significant blood loss and insufficient erythropoietin production associated with increased hepcidin.

Mild forms of iron deficiency and mixed anaemia are present in severly obese patients, before and after bariatric surgery. Athletes, especially endurance athletes, and mainly females, may present with iron deficiency caused by concomitant conditions, insufficient iron absorption, training, inflammation, gastrointestinal bleeding and iron loss in sweat during competitions.

Management of iron deficiency

Management of absolute iron deficiency entails: (i) identification and treatment of the underlying cause and (ii) correction of the deficiency by therapy. Iron deficiency is commonly due to blood loss and, wherever possible, the site of this must be identified and the lesion treated. Functional iron deficiency usually reverts with successful treatment of the underlying disorder. When not, and especially in cancer, erythropoietin or iron may be options.

Oral iron therapy

In most patients with absolute iron deficiency, body stores of iron can be restored by oral iron therapy based on ferrous iron salts; ferrous sulfate, being the cheapest, is usually the drug of first choice: 200 mg of ferrous sulfate contains 67 mg of iron, 300 mg of ferrous gluconate provides 36 mg of iron. Liquid iron preparations are available for infants and children.

Traditional therapy suggested high dose (100–200 mg/day) elementary iron to overcome the limited absorption and administration in refracted (2–3) doses. Reevaluation of this therapeutic approach has shown that hepcidin increases after each iron dose, suggesting that changing from daily to alternate-day schedules and from divided to single morning doses increases the efficacy and reduces side effects of oral treatment. In iron deficiency without or with mild anaemia low dose (60–120 mg) iron salts in the morning of alternate day may be considered an effective treatment. In severe anaemia, higher and daily doses restore haemoglobin faster.

Oral therapy may cause gastrointestinal side effects due to local iron toxicity of non-absorbed iron. Nausea, epigastric pain, diarrhoea and constipation are dose-related and can usually be ameliorated by reducing the dose or taking the iron with food, but this also reduces the amount absorbed. Enteric-coated and sustained-release preparations should not be used, as much of the iron is carried past the duodenum to sites of poor absorption. Iron reduces absorption of tetracyclines (and vice versa) and of ciprofloxacin.

The minimum rate of response should be a 20 g/L rise in haemoglobin every 3 weeks, and the usual rate is 1.5–2.0 g/L daily. This will be slower when the dose tolerated is less than 100 mg/day, but this is seldom of clinical importance. It is usually necessary to give iron for 3–6 months to correct the deficit of iron in circulating haemoglobin and in stores (shown by a rise in serum ferritin to normal).

Failure to respond to oral iron is most commonly due to the patient not taking the medication, although there may be continued haemorrhage or malabsorption. Refractoriness to oral iron is defined as <10 g/L haemoglobin increase after 4 weeks of treatment. In non-responding patients, it is important to reassess the diagnosis to exclude other causes of microcytic anaemia and iron-loading anaemias (see Chapter 14).

A poor response is usually observed in case of anaemia other than iron deficiency anaemia. In anaemia of inflammation, oral iron has limited role since when levels of serum hepcidin are high intestinal iron absorption is strongly reduced.

Oral iron may be used for prevention in areas where iron deficiency anaemia is endemic. Protocols suggested by WHO

are available: for example, low dose oral (30–60 mg elemental iron) twice a week for 3 months/year. Iron supplementation in children in areas endemic for malaria may worsen the infection, especially cerebral malaria, and should be associated with malarial treatment.

Parenteral iron therapy

This is indicated in subjects who genuinely cannot tolerate oral iron, particularly if gastrointestinal disease, such as inflammatory bowel disease, is present. It is also necessary for gluten-induced enteropathy and when it is essential to replenish body stores rapidly (e.g. where severe iron deficiency anaemia is first diagnosed in the second-third trimester of pregnancy) or when oral iron cannot keep pace with continuing haemorrhage (e.g. in patients with hereditary haemorrhagic telangiectasia).

When iron is used in functional iron deficiency or in mixed anaemia, parenteral preparations may be indicated because high levels of hepcidin cause refractoriness to oral therapy. Patients with chronic kidney disease who are being treated with recombinant erythropoietin also require parenteral iron therapy. Increased red cell loss at dialysis contributes to iron needs and oral iron therapy is usually inadequate to prevent an impaired response to erythropoietin. High hepcidin levels because of reduced clearance and inflammation contribute to erythropoietin resistance in renal failure. This occurs in other chronic inflammatory conditions: for example, the anaemia of cancer when treated with erythropoietin requires parenteral iron as well. Intravenous iron is indicated in flare-ups of inflammatory bowel diseases to avoid further intestinal mucosal damage by non-absorbed oral iron. Intravenous iron has been shown to improve functional capacity and quality of life in patients with congestive heart failure and iron deficiency, even in the absence of anaemia.

From all parenteral preparations, the iron complex is taken up by macrophages, from which iron is released to circulating transferrin, which then transports it to the marrow. High-molecular weight iron dextran has been abandoned because of potentially severe side-effects, including anaphylaxis. An iron–sucrose complex, Venofer, is given by intravenous infusion to a maximum of 200 mg in 1–2 hours and not repeated in less than 48 hours. The deficit in body iron should be calculated from the degree of anaemia; it is usually 1–2 g. In patients receiving erythropoietin treatment in chronic kidney disease, smaller intravenous doses of Venofer (25–150 mg/week) may be used, with regular monitoring of serum ferritin to avoid iron overload. Other compounds can provide all necessary iron (up to 1000 mg) in one infusion. Amongst these low-molecular-weight iron dextran, available in the UK as Cosmofer ferric derisomaltose (once isomaltoside) 1000 and ferricarboximaltose (Ferrinject), a macromolecular iron(III)-hydroxide carbohydrate complex (molecular weight approximately 150 kDa). The latter can be administered as slow infusion (maximum single dose 1000 mg over 15 min). These compounds have an improved safety profile and may be indicated in selected patients who have limited hospital access, when is urgent to restore haemoglobin, as in pregnancy, or to replete the exhausted stores. Headache, flushing, nausea, skin rashes, urticaria, shivering, general aches and pains, dyspnoea and syncope remain possible immediate adverse effects of intravenous iron. Delayed reactions including arthralgia, fever and lymphadenopathy can persist for several days and are treated with paracetamol. Administration by trained personnel and adherence to protocols to recognise and promptly manage reactions are advised.

Other rare anaemias due to alterations in iron metabolism are discussed in Chapter 12 since these are usually associated with iron overload.

Pathological alterations in haem synthesis

Porphyrias

These are a group of inherited or acquired diseases, each characterised by a partial defect in one of the enzymes of haem synthesis (Chapter 3) that may present as acute or chronic diseases. Increased amounts of the intermediates of haem synthesis accumulate, the disorders being classified by whether the effects are predominantly in the liver or the erythron (Table 11.6). A full discussion of these disorders is beyond the scope of this chapter, but those with a particular haematological overlap are mentioned briefly.

Congenital erythropoietic porphyria

This is a very rare autosomal recessive disorder that is due to reduced uroporphyrinogen III synthase activity. Most patients are compound heterozygotes for mutations in the uroporphyrinogen III synthase gene. In rare cases, a germline mutation of the X-linked erythroid-specific transcription factor GATA1 has been described. In uroporphyrinogen III synthase deficiency, large amounts of porphyrinogens accumulate, and their conversion by spontaneous oxidation to photoactive porphyrins leads to severe, and disfiguring, cutaneous photosensitivity and dermatitis, as well as a haemolytic anaemia with splenomegaly. Increased amounts of uroporphyrin and coproporphyrin, mainly type I, are found in bone marrow, red cells, plasma, urine and faeces. The age of onset and clinical severity of the disease are highly variable, ranging from non-immune hydrops fetalis to a later onset in which there are only cutaneous lesions. Treatment, including avoidance of sunlight and splenectomy to improve red cell survival, is only partially effective. High-level blood transfusions to suppress erythropoiesis (combined with iron chelation therapy) have been used to reduce porphyrin production sufficiently to abolish the clinical symptoms. Allogeneic bone marrow transplantation is indicated in severe cases.

Table 11.6 Human porphyrias.

Form	Inheritance	Enzyme defect	Clinical features*
Hepatic			
Acute intermittent porphyria	Autosomal dominant	Porphobilinogen deaminase	A
Hereditary coproporphyria	Autosomal dominant	Coproporphyrinogen oxidase	A + P
Porphyria variegate	Autosomal dominant	Protoporphyrinogen oxidase	A + P
Porphyria cutanea tarda	Acquired or (rare) autosomal dominant	Uroporphyrinogen decarboxylase	P
Erythropoietic			
Congenital erythropoietic porphyria	Autosomal recessive	Uroporphyrinogen cosynthase	P
Erythropoietic protoporphyria	Autosomal dominant	Ferrochelatase	P
X-linked protoporphyria		Rarely ALAS2	

* Acute attacks (A) of the gastrointestinal and/or nervous system are related to the accumulation of porphyrin precursors (δ-aminolevulinic acid and porphobilinogen). Photosensitive skin lesions (P) are seen when the level of the enzyme defect in the haem synthetic pathway leads to the accumulation of formed porphyrins.

Erythropoietic protoporphyria

This is the most common erythropoietic porphyria and is usually caused by an autosomal dominant inherited deficiency of ferrochelatase, which results in increased free (not Zn-linked) protoporphyrin concentrations in bone marrow, red cells, plasma and bile. Bone marrow reticulocytes are the primary source of the excess protoporphyrin. This leaks from cells and is excreted in the bile and faeces. In >90% of patients, EPP results from coinheritance of a rare pathogenic variant in trans of a common hypomorphic variant c.315-48T>C (minor allele frequency 0.05). The onset of the disease is usually in childhood.

Expression of ferrochelatase is variable, and photosensitivity and dermatitis range from mild or absent to moderate in degree. The disease often remains undiagnosed. There is little haemolysis, but mild hypochromic anaemia may occur, and accumulation of protoporphyrins can occasionally lead to severe liver disease. Treatment is by the avoidance of sunlight; β-carotene may also diminish photosensitivity. Iron deficiency should be avoided as this may increase the amount of free protoporphyrin.

Rare patients with a variant, X-linked erythropoietic protoporphyria, have a gain-of-function mutation of *ALAS2*, usually responsible for X-linked sideroblastic anaemia (see below). Truncations of *ALAS2* C-terminal inhibitory domain lead to erythropoietic protoporphyria because upregulated *ALAS2* activity causes excessive protoporphyrin IX production.

Porphyria cutanea tarda

This is the most common of the hepatic porphyrias and occurs worldwide. The incidence in the UK has been estimated at 2–5 per million. 'Sporadic' porphyria cutanea tarda (PCT) accounts for about 75% of cases. The underlying metabolic abnormality is decreased activity of uroporphyrinogen decarboxylase (UROD) in the liver. Familial PCT accounts for about 25% of cases and is either an autosomal dominant disorder caused by mutations in the *UROD* gene or in few cases result from unknown inherited defects that affect hepatic UROD activity. There is a marked increase in porphyrins in liver, plasma, urine and faeces. The disease is characterised by photosensitivity and dermatitis. It is precipitated in middle or later life, more often in men than women, by factors such as liver disease, alcohol excess, hepatitis C or HIV infection, smoking or oestrogen therapy. A modest increase in liver iron is a common feature. Susceptibility to develop hepatocellular carcinoma is reported.

Either the homozygous or heterozygous presence of the C282Y and H63D mutations in the *HFE* gene (Chapter 12) may predispose to the development of PCT. Prevalence of the C282Y mutation is increased in both sporadic and familial PCT. In the UK, only homozygosity for C282Y (found in about 25% of patients) is significantly more common than in the general population (0.7%). In southern Europe, where C282Y is much less common, the H63D mutation is associated with PCT. Iron is known to inhibit UROD. Removal of the iron by repeated phlebotomy is standard treatment, usually leading to remission. Iron chelation is used when phlebotomy is contraindicated. Sun exposure should be avoided. Antimalarial agents, as chloroquine or hydroxychloroquine, have some effects on skin lesions, although the mechanism is unclear.

Homozygosity or compound heterozygosity for *UROD* mutations causes hepatoerythropoietic porphyria with early onset of both erythroid and cutaneous manifestations, similar to congenital erythropoietic porphyria.

Lead poisoning

Chronic ingestion of lead in humans causes an anaemia that is usually normochromic or slightly hypochromic. Red cell lifespan is shortened and there is a mild increase in reticulocytes, but jaundice is rare. Basophilic stippling is a characteristic, though not universal, finding and it is thought to be due to precipitation of RNA, resulting from inhibition of the enzyme pyrimidine 5′-nucleotidase. Siderotic granules, and occasionally Cabot rings, are found in circulating red cells. The bone marrow shows increased sideroblasts, in some patients with ring sideroblasts. Red cell protoporphyrin and coproporphyrin are raised, as is urinary excretion of ALA, coproporphyrin III and uroporphyrin I.

The cause of the anaemia appears to be multifactorial. Haemolysis, probably due to the blocking of sulfydryl groups with consequent denaturation of structural proteins, and damage to mitochondria, with defective haemoglobin production due to inhibition of the enzymes of haem synthesis are the major factors.

Sideroblastic anaemia

The sideroblastic anaemias comprise a group of refractory anaemias (Table 11.7) in which there are variable numbers of hypochromic cells in the peripheral blood, with an excess of iron in the bone marrow; at least 15% of the developing erythroblasts in the primary forms of anaemia contain iron granules arranged in a ring around the nucleus (Figure 11.7). These ring sideroblasts (more than four perinuclear granules per cell and covering one-third or more of the nuclear circumference) are the diagnostic feature of the anaemia. Ring sideroblasts may comprise a small (<15%) population of the erythroblasts in a wide variety of clinical disorders. SA can be inherited or acquired (Table 11.7).

Table 11.7 Classification of sideroblastic anaemias.

Hereditary non syndromic	Inheritance	Anaemia type
ALAS2 mutations	X-linked	Microcytic
Mitochondrial glycine importer *(SLC25A38)* mutations	Recessive	Microcytic
ABCB7 (mitochondrial ATP-binding cassette) mutations; spinocerebellar ataxia	X-linked	Microcytic
Glutaredoxin-5 (*GLRX5)* mutations	Recessive	Microcytic
Heat shock protein (HSPA9) deficiency	Recessive	Normo/microcytic
Heat shock cognate protein (HSC20) deficiency	Recessive	Normocytic
Hereditary syndromic		
NDUFB11 (subunit of respiratory complex I) mutations	X-linked	Normocytic
MLASA I pseudouridine synthase-1 *(PUS1)* mutations; mitochondrial myopathy	Recessive	Normo/macrocytic
MLASA II *YARS2* mutations, mitochondrial myopathy	Recessive	
THTR-1 (*SLC19A2*) mutations, DIDMOAD syndrome, Thiamine responsive	Recessive	Macrocytic
tRNA-nucleotidyltransferase1(*TRNT1)* mutations; with SIFD: immunodeficiency, periodic fever and developmental delay	Recessive	Microcytic
Mitochondrial DNA deletion: Pearson (marrow–pancreas) syndrome	Sporadic/mitochondria	Macrocytic
Acquired		
Primary		
Refractory anaemia (myelodysplasia) with ring sideroblasts (RARS)	Somatic mutations	Macrocytic
Secondary		
Drugs: isoniazid, pyrazinamide, cicloserine, chloramphenicol, penicillamine	NA	Normo/macrocytic
Mitochondrial toxicity: alcohol, lead poisoning	NA	Macrocytic
Copper deficiency in carcinoma, rheumatoid arthritis	NA	Normo/macrocytic

DIDMOAD, diabetes insipidus, diabetes mellitus, optic atrophy and deafness; MLASA, myopathy, lactic acidaemia, sideroblastic anaemia; SIFD, sideroblastic anaemia, B-cell immunodeficiency, periodic fevers, developmental delay.

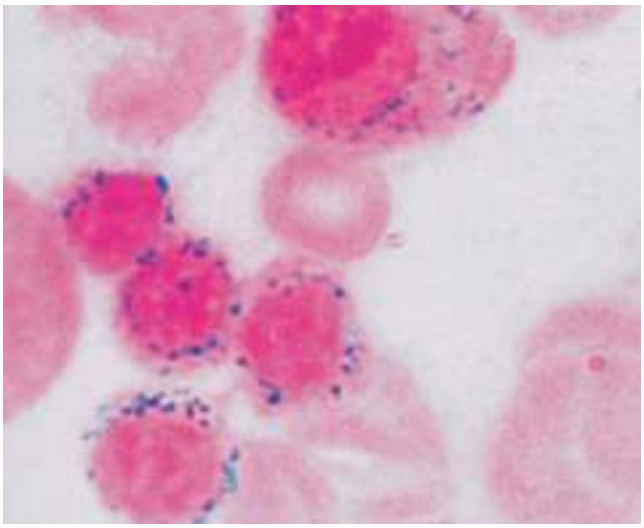

Figure 11.7 Sideroblastic anaemia. Erythroblasts showing perinuclear (mitochondrial) rings of iron (Perls' stain).

Inherited sideroblastic anaemias

These are rare disorders manifesting usually in childhood or adolescence, but occasionally presenting late in life, when they need to be distinguished from the more common acquired form termed 'refractory anaemia with ring sideroblasts'. Inherited sideroblastic anaemias may show exclusively anaemia as the main symptom or present within a syndrome that includes anaemia in the context of other non-haematologic manifestations (syndromic sideroblastic anaemia) (Table 11.7). This is because they may affect proteins relevant only in erythropoiesis or in several other tissues because are proteins of mitochondrial translation, or a component of the mitochondrial respiratory chain. A detailed discussion of syndromic forms is beyond the scope of this chapter; their haematologic features are mentioned.

Defective haem synthesis

X-linked sideroblastic anaemia (*ALAS2* mutations)

In most reported families, inheritance follows an X-linked pattern. More than 100 different mutations of the gene for erythroid-specific *ALAS2* have been identified. All have been single-base substitutions, mostly missense mutations, that lead to changes in protein structure, causing instability or loss of function. They are found scattered over the exons encoding the catalytically active domain of the protein. Mutations affecting the promoter have also been shown to cause disease. Function may be rescued to a variable degree by administration of pyridoxal phosphate (the essential cofactor for *ALAS2*), the best responses occurring when the mutation affects the pyridoxal phosphate-binding domain of the enzyme. The response is better if iron overload is reduced by phlebotomy or chelation.

Female carriers of X-linked sideroblastic anaemia may show partial haematological expression, usually with only mild or no anaemia, although rarely a severe dimorphic anaemia occurs. This may depend on variation in the severity of the defect, as well as the degree of lyonisation of the affected X chromosome. Late-onset in some patients is observed since the degree of lyonisation increases with age. Iron loading may also aggravate the defect in haem synthesis in both males and females with sideroblastic anaemia.

Patients with X-linked sideroblastic anaemia show a hypochromic, often microcytic, anaemia. There may be a few circulating siderocytes, normoblasts and cells with punctate basophilia, but these features become pronounced only if the spleen has been removed. The bone marrow shows erythroid hyperplasia and the erythroblasts tend to be microcytic with a vacuolated cytoplasm. Ring sideroblasts account for more than 15% of erythroblasts. The ineffective erythropoiesis is not usually accompanied by bone deformities, but some bossing of the skull and enlargement of the facial bones may result from the erythroid expansion. The spleen may be enlarged. Patients may present with severe iron overload even when the anaemia is relatively mild, but the rate of iron loading is accelerated if red cell transfusions are needed. Iron loading, however, aggravates anaemia, while iron reduction improves this.

A form of X-linked sideroblastic anaemia is seen due to mutations in the ATP-binding cassette transporter gene (*ABCB7*). It also causes early-onset, non-progressive cerebellar ataxia. The anaemia is mild to moderately severe. The enzyme deficiency induces disruption in the maturation/export of iron–sulfur clusters to cytosol. The ataxia may be due to iron damage to mitochondria in neural cells.

Mutations of mitochondrial glycine importer (*SLC25A38*)

This is the second most frequent form of sideroblastic anaemia. The mitochondrial Solute Carrier Family 25 Member 38 carrier (*SLC25A38)* encodes an erythroid-specific transporter involved in glycine import from cytosol to the mitochondria, an essential step in synthesis of ALA. Mutations in congenital sideroblastic anaemia are described in >50 families from different ethnic groups with recessive inheritance. The degree of anaemia, which is microcytic and hypochromic, is usually severe since infancy and transfusion-dependent. Some patients have been cured by allogeneic bone marrow transplantation.

Mutations of proteins of the iron–sulfur cluster biogenesis

Glutaredoxin 5 *(GLRX5)* participates in iron–sulfur cluster mitochondrial trafficking. A few families worldwide have been described with autosomal recessive hypochromic microcytic anaemia with ring sideroblasts with inherited mutations of the enzyme. Other rare forms of sideroblastic anaemia are due to mutations of proteins of iron–sulfur cluster pathway, as heat

shock protein (HSP)A9 or heat shock cognate protein 20 (HSC20) that with *GLRX5* facilitate the transport of nascent 2-iron/2sulfur clusters to recipient mitochondria proteins. The common molecular pathogenic mechanism is the reduction of iron–sulfur cluster for ferrochelatase and other enzymes of haem biosynthesis, which underline how the two pathways of iron utilisation by mitochondria are interconnected.

Syndromic forms of sideroblastic anaemia

These forms are due to defective proteins relevant in mitochondrial translation or respiratory chain function. Patients present multiple and variable phenotypes since infancy, usually severe and in some cases even lethal. The main forms are described here.

Associated with mitochondrial myopathy

Mitochondrial myopathy, lactic acidosis and sideroblastic anaemia (MLASA) result from a homozygous mutation in the nuclear-encoded gene for pseudouridine synthase. As in Pearson syndrome (see mitochondrial DNA mutations), there are defects of the mitochondrial electron transport chain affecting reduced access to ferrochelatase. Mutations affecting the pseudouridylate synthase 1 (*PUS1*) gene cause deficient pseudouridylation of mitochondrial tRNAs. Mutations of the *YARS2* gene product, mitochondrial tyrosyl-tRNA synthetase may cause the same MLASA phenotype through respiratory chain dysfunction.

Associated with immunodeficiency

A syndromic form of recessive sideroblastic anaemia leads to immunodeficiency, periodic fevers, and developmental delay (SIFD); it is caused by mutations in transfer RNAs (tRNA)-nucleotidyltransferase 1 (*TRNT1*), the gene encoding the enzyme adding the nucleotide sequence CCA to the 3′ end of tRNA, essential for maturation of both nuclear and mitochondrial tRNAs.

Associated with megaloblastic anaemia

Abnormalities of *SLC19A2* gene encoding a putative thiamine transporter (THTR-1) are responsible for thiamine-responsive megaloblastic anaemia (Roger syndrome). This syndrome, inherited in an autosomal recessive manner, may also combine diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD), which respond in varying degrees to pharmacological doses of thiamine (vitamin B_1). Ring sideroblasts in varying numbers are typically present and onset is usually in childhood, although some symptoms may be present in infancy. A direct link between the presence of the mutation and mitochondrial iron loading has yet to be demonstrated. High doses of thiamine (vitamin B1; 25–75 mg per day) are administered to alleviate anaemia and possibly improve diabetes control.

Defective mitochondria respiratory chain

A rare form is due to NADH ubiquinone oxidoreductase subunit B11 (*NDUFB11)* mutations. The gene encodes a component of the mitochondrial complex 1 of the respiratory chain. A recurrent mutation is responsible for variably syndromic, normocytic normochromic sideroblastic anaemia. Hemizygosity for a null mutation is likely lethal. The partial inactivation of a recurring mutation causes a mitochondrial respiratory phenotype such as lactic acidosis and myopathy, which will distinguish this disorder from other CSAs.

Deletions of mitochondrial DNA, sometimes associated with duplications, are known to be the cause of the Pearson marrow–pancreas syndrome, typically consisting of sideroblastic anaemia, pancreatic exocrine dysfunction and lactic acidosis. This is a severe disorder of early onset, presenting usually with failure to thrive, persistent diarrhoea and lactic acidosis. All haemopoietic cell lineages can be affected, and the anaemia is typically macrocytic with prominent vacuoles in cells of both myeloid and erythroid lineages.

Mitochondrial DNA encodes mitochondrial tRNA and ribosomal RNA as well as several mitochondrial proteins. In Pearson syndrome, deletions may encompass tRNA as well as mitochondrial genes and therefore have an effect on the function of all mitochondrion-encoded proteins, causing considerable loss of mitochondrial function. The presence of many different mitochondria within nucleated cells enables the coexistence of normal and abnormal species, the proportion of which is likely to vary within different tissues, a phenomenon known as *heteroplasmy*. The extent to which different tissues are affected depends to some extent on this proportion and detection often requires the study of different tissues. Inheritance is difficult to determine for the same reason and most cases are described as of 'sporadic' occurrence. There is no specific treatment.

There are still a limited number of cases of inherited sideroblastic anaemia in which the exact underlying genetic defect remains obscure, with variable inheritance and often a macrocytic or dimorphic picture.

Acquired sideroblastic anaemia

Refractory anaemia with ring sideroblasts

This is a form of myelodysplasia and arises as a clonal disorder of haematopoiesis (see also Chapter 35). According to the last nomenclature, it is classified as a low-risk myelodysplsia with ring sideroblasts (MDS-RS) with uni-lineage or multi-lineage involvement. The anaemia is often macrocytic with raised red cell protoporphyrin concentrations, in contrast to X-linked sideroblastic anaemia. Marked erythroid hyperplasia may be present, together with increased iron stores. In these low-risk patients, transformation to acute myeloid leukaemia appears less than in other myelodysplastic disorders. Somatic mutations

may be present as in other clonal forms; mutations of the spliceosome *SF3B1* gene have been found in more than 90% of patients with refractory anaemia with ring sideroblasts (once called RARS) and allow diagnosis by molecular testing (Chapter 34). Smaller numbers (<15%) of ring sideroblasts may be present in patients with any of the other myelodysplastic and myeloproliferative diseases. Acquired defects of mitochondrial DNA may underlie iron transport abnormalities in refractory anaemia with ring sideroblasts.

Secondary sideroblastic anaemias

Sideroblastic anaemia may be found as a complication of antituberculous chemotherapy, particularly with isoniazid and cicloserine (pyridoxine antagonists). Sideroblastic anaemia occurs in alcohol use disorder if there is associated malnutrition and folate deficiency. Suggested mechanisms include interference with haem formation and pyridoxine metabolism. The anaemia rapidly reverses with abstinence from alcohol, a normal diet and pyridoxine therapy. Chloramphenicol inhibits mitochondrial protein synthesis and, in some patients, causes ring sideroblast formation, presumably as a result of impaired haem formation in the mitochondria. Lead inhibits several enzymes involved in haem synthesis and may damage structural mitochondrial proteins. In some cases, ring sideroblasts are visible in the marrow. Ring sideroblasts may also occur in erythropoietic porphyrias and have been rarely documented in severe iron-loaded beta-thalassaemia.

Treatment

A proportion of patients with X-linked sideroblastic anaemia respond to pyridoxine, given initially in doses of 100–200 mg daily. The response is usually partial. Patients may require only small doses (less than 10 mg daily) to maintain a higher haemoglobin concentration. Pyridoxine therapy is ineffective in RARS. However, some secondary sideroblastic anaemias may be completely reversed by pyridoxine therapy. This has been described in alcohol excess, haemolytic anaemia and gluten-induced enteropathy, as well as in patients receiving antituberculous chemotherapy, in whom the drugs have been stopped and pyridoxine administered.

Folic acid may benefit patients with secondary sideroblastic anaemia. For refractory patients, the anaemia may remain stable and, if the patient is transfusion independent, no treatment is needed. Patients under regular red cell transfusions require iron chelation therapy (see Chapter 12). Iron loading may aggravate the anaemia and, in some patients, improvement in the anaemia has followed iron removal by phlebotomy or iron chelation therapy. Splenectomy should usually be avoided as it does not benefit the anaemia and leads to persistently high platelet counts postoperatively, with a high incidence of thromboembolism. Bone marrow transplantation may be proposed for severe non-syndromic forms of sideroblastic anaemia as *SLC25A38* deficiency. The activin ligand trap, luspatercept reduces transfusion requirement in a proportion of acquired forms and is now approved for the treatment of transfusion-dependent patients with MDS and ring sideroblasts refractory to erythropoiesis-stimulating agents.

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CHAPTER 12

Iron overload

12

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Introduction

Excessive iron accumulation may lead to tissue damage. Iron overload of the parenchymal cells of the liver, endocrine organs and the heart may arise when there is excessive iron absorption, whereas iron administered parenterally (e.g. as multiple transfusions) is first taken up by macrophages, which destroy senescent red cells. However, there is no absolute distinction between the two sources of iron loading, as iron in macrophages is slowly released to transferrin from which it is taken up by parenchymal cells. The causes of iron overload are shown in Table 12.1. Severe iron overload, arbitrarily defined as an excess of storage iron of more than 5 g, is confined to the hereditary forms of haemochromatosis and the iron-loading anaemias.

Hereditary haemochromatosis

Hereditary haemochromatosis (HH) is now classified according to the genetic defect causing iron overload (Table 12.2). The vast majority of cases are of Type 1, involving the *HFE* gene (Chapter 11). In populations of northern European origin, most patients with HH are homozygous for the *HFE* pCys282TyrC mutation (pC282Y). All the other types, often indicated as non-HFE HH, are very rare. Types 1, 2 and 3 HH are autosomal recessive diseases and share common features due to hepcidin deficiency, including high transferrin saturation and hepatocyte iron accumulation. Type 4 HH, inherited as a dominant condition, is a heterogeneous disease with variable clinical phenotype.

HFE haemochromatosis

HFE HH is one of the most common genetic conditions in populations of northern European origin, associated with mutations in the *HFE* gene.

The *HFE* gene is an atypical MHC class I-like gene located at 6p21. The association of HH with HLA-A3 suggested a founder mutation in a chromosome carrying the A3 haplotype. Homozygosity for a G→A substitution at nucleotide 845 of the *HFE* gene results in a cysteine to tyrosine substitution at amino acid 282 (pC282Y). A second variant (187C→G) results in a histidine to aspartic acid substitution at amino acid 63 (pHis63Asp or pH63D).

HFE genotypes have been reported from large population studies throughout the world. In a study of 100,000 multiethnic participants from primary care practices, the estimated prevalence of the pC282Y homozygous genotype was higher in white (1 in 227, 0.44%) than in black individuals and was extremely rare in Asian people. The frequency is higher in northern Europe than in Italy and Greece. In the UK, about 1 in 8 people are carriers of the pC282Y mutation, and about 1 in 200 are homozygous for this mutation. Homozygosity is strongly associated with HH, with about 90% of patients with genetic iron overload having this genotype in northern Europe and about 60% in southern Europe. The HH gene may have increased in frequency because of a selective advantage for heterozygotes and protection against iron deficiency anaemia. Homozygotes would be unlikely to suffer the effects of iron overload before reproducing.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 12.1 Causes of iron overload.

Genetic iron overload
Hereditary haemochromatosis
Atransferrinaemia
DMT1 deficiency
Acaeruloplasminaemia
Secondary iron overload
Iron-loading anaemias (ineffective erythropoiesis)
(e.g. β-thalassaemia intermedia, sideroblastic anaemia, congenital dyserythropoietic anaemia)
Repeated red cell transfusions
congenital anaemias (e.g. β-thalassaemia major, sickle cell anaemia, Blackfan–Diamond anaemia)
acquired anaemias (e.g. MDS, aplastic anaemia, allogeneic bone marrow transplantation)
Other causes of iron overload
Neonatal haemochromatosis
Chronic liver diseases
African iron overload

The pH63D mutation is found throughout the world with a mean allele frequency of 15%. A small proportion of HH patients are compound heterozygotes for the pC282Y and pH63D mutations. Other *HFE* gene mutations/deletions associated with iron accumulation have been described, mostly in individual families.

That *HFE* was the HH gene was confirmed by the demonstration that *HFE* knockout mice and mice homozygous for the pC282Y mutation develop iron overload. In addition, tissue-specific inactivation of HFE protein in hepatocytes, but not in duodenal cells or macrophages, leads to iron overload, indicating a specific function of HFE in the liver. A model of the pathophysiology of the disease suggests that HFE competes with diferric transferrin for TFR1 binding and that when diferric transferrin binds TFR1, liver HFE binds TFR2, thus forming a complex that activates hepcidin synthesis (Chapter 11). Low serum hepcidin explains the findings in the early stages of HH: increased iron absorption, a raised serum iron and transferrin saturation, iron accumulation in hepatocytes with a periportal distribution and a paucity of iron in macrophages.

Iron parameters and clinical status of patients with *HFE* mutations

Transferrin saturation and serum ferritin are significantly higher in pC282Y/pC282Y subjects than in other genotypes. Increased levels of iron parameters are present in 75–100% of males and 40–60% of females in different studies. In population surveys slightly, but significantly, higher values for serum iron and transferrin saturation have been found in heterozygotes for either pC282Y or pH63D compared with subjects lacking these mutations. The differences in serum ferritin levels are small and not significant, except in rare cases where mutations in other genes involved in iron metabolism may be present. In compound heterozygotes, and those homozygous for pH63D, significant iron accumulation is rare and liver fibrosis exceptional. The frequency of homozygosity or heterozygosity for pC282Y or pH63D mutations is not increased in patients with arthritis, diabetes and heart disease.

In pC282Y, homozygotes increased non-transferrin-bound iron (NTBI) is taken up by several organs. A gradual accumulation of iron may lead to tissue damage, which may present as cirrhosis of the liver, diabetes, hypogonadism, cardiomyopathy, arthritis and a slate-grey skin (melanin) pigmentation. Only the most severe forms develop cardiac involvement that in the early

Table 12.2 Classification of hereditary haemochromatosis.

Type	Gene	Inheritance and phenotype	Severity	Incidence
1	*HFE*	AR, parenchymal iron overload	Highly variable	Common
2 (Juvenile)	Haemojuvelin (*HJV*)	AR, parenchymal iron overload	Severe	Rare
	Hepcidin (*HAMP*)	AR, parenchymal iron overload	Severe	Rare
3	*TFR2*	AR, parenchymal iron overload	Variable	Rare
4a Ferroportin disease	*SLC40A1* loss of function mutations	AD, RE iron overload	Variable	Rare
4b Dominant Haemochromatosis*	*SLC40A1* gain of function mutations (in binding site for hepcidin)	AD, parenchymal iron overload	Severe	Rare

* haemochromatosis due to hepcidin-resistant ferroportin
AD, autosomal dominant; AR, autosomal recessive; RE, reticuloendothelial. For gene symbols see Table 12.1.

stages is characterised by diastolic dysfunction and arrhythmias, in the later stages by dilated cardiomyopathy. Hepatocellular carcinoma may develop up to 25% of established cases with cirrhosis. Most patients present between the ages of 40 and 60 years; menstrual losses and pregnancies account for a generally delayed onset in women. Thus the clinical penetrance is low, estimated around 25% in males and 1% in females. The *HFE* pC282Y homozygous genotype only confers susceptibility to develop iron overload. Full phenotypic expression of the disorder is dependent on environmental factors, including dietary iron intake, blood donations or blood loss and other genetic factors modifying the genotype, although the latter effect is mild. Alcohol is a definite risk factor for the development of cirrhosis in patients homozygous for pC282Y.

Subjects homozygous for *HFE* pC282Y compared with control subjects with normal *HFE* genotype have shown the same frequencies of haemochromatosis symptoms and complications such as lethargy, arthralgia and diabetes. There is, however, a small but significant increase in subjects with either raised serum transaminases or fibrosis/cirrhosis in the pC282Y homozygous group. Homozygosity for pC282Y is more frequent in patients with cirrhosis and hepatoma than in the general population. The relative risk for hepatocellular carcinoma in cirrhotic patients with HH is approximately 20, with an annual incidence of 3–4%. Monitoring the serum α-fetoprotein is needed in both untreated and treated cases, because risk of liver cancer remains also in iron-depleted cirrhotic patients.

There is evidence that ferritin concentration above 1000 μg/L is a risk factor for liver fibrosis with a prevalence of 20–45%. A serum ferritin level >1000 μg/L has 100% sensitivity and 70% specificity for identification of cirrhosis. The ferritin cut-off might be lower in the presence of cofactors for liver disease such as alcohol. Patients with serum ferritin level >1000 μg/L should undergo liver biopsy or magnetic resonance imaging (MRI) for iron quantitation and Fibroscan for fibrosis evaluation (see below) and complete cardiac and endocrinological evaluation. Only about 50% of homozygous women have raised transferrin saturation and progression through iron accumulation and tissue damage is usually, but not always, slower.

Population surveys have shown that less than 5% of subjects homozygous for pC282Y ever receive a diagnosis of HH. Most men who are homozygous for pC282Y will have a raised transferrin saturation before the age of 30 years; a proportion will have an elevated serum ferritin concentration, but only a minority will eventually develop fibrosis and cirrhosis of the liver. Studies in Australia and Norway found that 5% of homozygote males, but no females, showed cirrhosis. Time-dependent ferritin increase is slow.

Clinical and laboratory diagnosis

Although the biochemical penetrance of pC282Y homozygosity is substantial (about 70%), the disease penetrance is significantly lower (about 25%) amongst men and (1%) amongst women.

Figure 12.1 Radiograph of hand: patient with haemochromatosis showing loss of joint space and erosion of cartilage at the metacarpophalangeal joints (arrow).

The variety of clinical presentations and their lack of specificity for HH mean that a high degree of clinical suspicion is needed. Fatigue, abdominal pain, diabetes mellitus, signs and symptoms of gonadal failure and arthritis may be present for several years before the diagnosis is made. Arthritis particularly affects the second and third metacarpophalangeal joints (Figure 12.1), and destructive arthropathy of hip and knee joints occurs in 10% of patients. There is chondrocalcinosis with pyrophosphate deposition in the joints. Abdominal pain may result from hepatic enlargement or hepatocellular carcinoma. Grey skin pigmentation results from excess melanin deposition.

In asymptomatic subjects, iron accumulation is indicated by a raised transferrin saturation (>45%). Most men and about 50% of women who are homozygous for *HFE* pC282Y will have a raised transferrin saturation. As iron accumulates, the serum ferritin concentration rises, and values in excess of 200 μg/L (women) and 300 μg/L (men) suggest iron overload. Serum ferritin concentrations largely reflect iron turnover in phagocytic cells and do not provide an early indication of iron accumulation

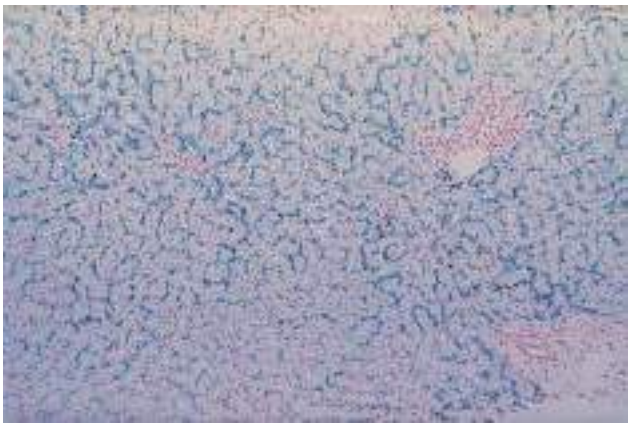
in liver parenchymal cells. Thus, measurement of transferrin saturation is essential for early detection of iron loading. However, in patients with infection, inflammation or malignancy or in those undergoing surgery, transferrin saturation may be depressed and the serum ferritin concentration elevated. In most cases, *HFE* genotyping will confirm the diagnosis of HH.

Testing adult first-degree relatives of a pC282Y homozygous patient may identify subjects homozygous for *HFE* pC282Y with normal serum ferritin concentration and without evidence of liver disease: they should have transferrin saturation and serum ferritin measured at yearly intervals. Compound heterozygotes are at lesser risk of iron overload but should also be tested by measuring iron parameters. Widespread population screening by iron status or genetic testing is considered unwarranted as the level of risk for a pC282Y homozygote developing iron overload appears to be low. Screening based on iron parameters is advisable in high-risk populations.

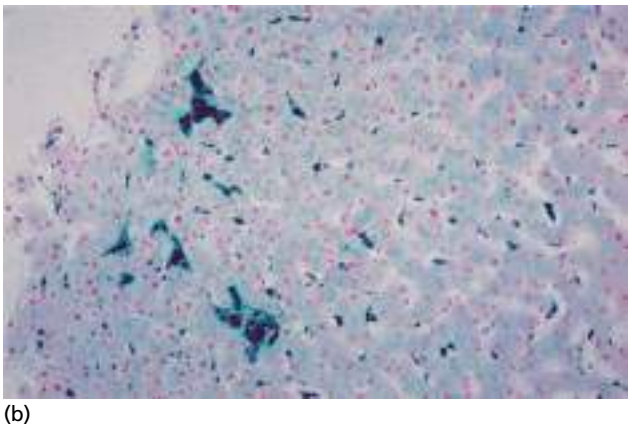
Associations with other conditions

The pC282Y mutation is relatively common (see above); heterozygosity, and even homozygosity, may occur with other haematological conditions, including inherited sideroblastic anaemia, β-thalassaemia trait or other haemolytic anaemias and aggravate a tendency to iron load. The mutation may worsen liver disease in conjunction with hepatitis C infection, alcohol and non-alcoholic fatty liver disease. Porphyria cutanea tarda, which may be associated with iron overload, an important trigger of disease manifestations, is discussed in Chapter 11.

The *HFE* pC282Y mutation was identified in multiple genome-wide association studies for quantitative loci as erythrocyte and iron traits, blood pressure and HbA1C levels in diabetics and others, likely through indirect effects or due to the association of the *HFE* gene with the HLA complex.



(a)



(b)

Figure 12.2 Liver histology (Perls' stain). (a) Liver biopsy from a patient with Type 1 haemochromatosis, showing staining predominantly in parenchymal cells. (b) Liver biopsy from a patient with ferroportin disease showing iron staining predominantly in Kupffer cells.

Body iron quantitation

Liver iron concentration is a good estimate of total body iron. Liver iron can be measured on a dried fragment of liver biopsy. The normal ranges are 0.17–1.8 mg/g dry weight. Values in excess of 3.0 mg/g dry weight indicate iron overload. Since the advent of genetic testing, liver biopsy is not usually performed. Iron quantitation in liver, heart and other organs is performed by non-invasive methods, mainly MRI techniques.

In patients homozygous for pC282Y with evidence of liver disease, hepatomegaly, increased transaminases and serum ferritin concentration >1000 μg/L, liver biopsy may be advisable to assess tissue damage. The biopsy reveals the hepatocyte iron accumulation with typical periportal predominance (Figure 12.2a). The amount of iron removed to reach iron depletion can be calculated (see Treatment) and provides a good estimate of total body iron. The amount of iron removed at each venesection is calculated by weighing the blood bag before and after venesection (density of blood is 1.05 g/mL) and assuming that 450 mL of blood (haemoglobin concentration 135 g/L) contains approximately 200 mg of iron. Iron absorption should be allowed at the rate of 3 mg daily (20 mg/week). With these assumptions, 25 weekly venesections will remove 4.5 g of iron. The amount of storage iron measured by the technique in normal adults has been shown to be about 750 mg in men and 250 mg in women.

Treatment

Although no randomised controlled trial is available to compare phlebotomy versus no phlebotomy in the treatment of HH, retrospective studies show that removal of excess iron by regular phlebotomy greatly reduces the morbidity and mortality from cardiac failure and diabetes complications. However, hepatocellular carcinoma accounts for a substantial proportion of deaths in those with established cirrhosis. Early diagnosis is therefore a priority, as patients identified and treated before the onset of cirrhosis and/or diabetes have a normal life expectancy.

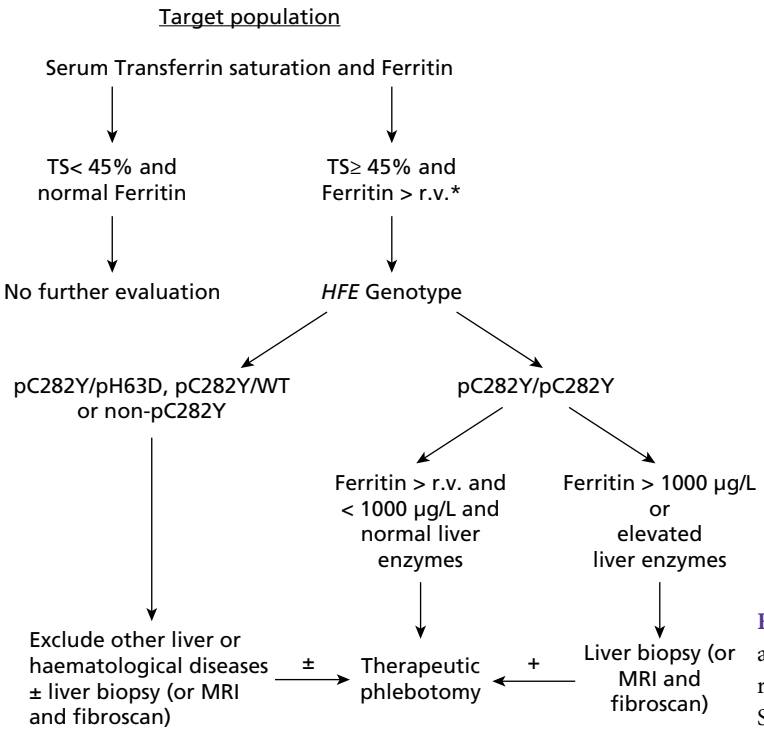


Figure 12.3 Algorithm for haemochromatosis diagnosis and treatment. The scheme is modified from the recommendations of the American Association for the Study of Liver Diseases (AASLD), and other scientific societies. TS, transferrin saturation; MRI, magnetic resonance imaging.

Since no test is available to identify those patients who will progress to fibrosis and those who will not, the present recommendation is to phlebotomise all patients with evidence of iron overload. No evidence-based guidelines for HH treatment by phlebotomy are available. Treatment is conventionally initiated when serum ferritin levels exceed the normal range. An algorithm for diagnosis and treatment of the disease based on current recommendations is shown in Figure 12.3.

Phlebotomy should be at a rate of 400–450 mL of blood each week and is performed until a target serum ferritin <100 µg/L is reached. Haemoglobin levels should be measured weekly and the rate of venesection reduced if anaemia develops. Serum ferritin should be monitored monthly. Weekly phlebotomy will need to be continued to remove total iron excess, which is usually greater than 5 g in established symptomatic disease, but may be >20 g. When iron stores are normalised, the frequency of phlebotomy should be reduced to two to four units each year, to continue indefinitely. The aim is to prevent re-accumulation of iron by maintaining a serum ferritin in the normal range (50–100 µg/L). The use of proton pump inhibitors has been shown to reduce the annual number of phlebotomy sessions. Fatigue, abdominal pain, transaminase elevation, mild fibrosis and skin pigmentation usually reverse on venesection. In some patients, diabetes mellitus, hypogonadism and arthralgia improve, but cirrhosis and destructive arthritis are irreversible. Early cardiac disease may respond to phlebotomy, while severe cardiomyopathy requires iron chelation (see below). Patients with end-stage liver disease and HCC may undergo orthotopic liver transplantation, after iron depletion. Post-transplantation survival rates in untreated patients are reduced compared with those of non-iron-loaded patients due to iron toxicity and infectious complications.

No diet recommendation is needed except limiting alcohol intake and avoiding raw shellfish for possible infection with *Vibrio vulnificus*, which is a particular risk in iron-loaded patients. This occurs because *Vibrio vulnificus* avidly takes up NTBI, the levels of which increase in patients with high transferrin saturation.

Iron chelation with subcutaneous desferrioxamine (DFO) may be used in patients who do not tolerate phlebotomy or have concomitant anaemia. DFO given as a continuous intravenous infusion with or without an oral iron chelator (see iron chelation therapy), an off-label treatment, may have a role in the short-term management of patients with life-threatening cardiac failure. Oral iron chelators such as deferasirox at low doses are likely to be used for selected patients in the future. Targeting the hepcidin pathway to increase hepcidin production ameliorates iron overload in preclinical models of the disease.

Non-HFE haemochromatosis

Non-*HFE* HH is a clinical definition applied to all the other types of HH (Table 12.2). These forms are all rare, accounting for <5% of all cases. In juvenile HH, clinical symptoms usually appear in the

second and third decades of life. The disease can be due either to haemojuvelin mutations, that strongly decrease hepcidin production (see Chapter 11), or, more rarely, to mutations in the hepcidin (*HAMP*) gene. In both cases iron absorption is greater than in *HFE* HH and iron deposition occurs not only in the hepatocytes, but also in cardiac myocytes, pancreas and pituitary; symptoms of cardiomyopathy, diabetes and hypogonadism are prominent (Table 12.2). HH Type 3 is phenotypically similar to *HFE* HH, although it may present at an earlier age. It is due to mutations in the gene that encodes the iron sensor transferrin receptor 2 (*TFR2*) (Table 12.2). All types of HH except Type 4 (Table 12.2) are recessive and characterised by low hepcidin secretion, the defect being the most severe in juvenile HH. Mutations in all of the genes of non-*HFE* HH (haemojuvelin, hepcidin, transferrin receptor 2 and ferroportin) have been found in rare patients from the Asia-Pacific region, where the *HFE* mutations are exceptional.

HH Type 4 is inherited as an autosomal dominant trait due to heterozygous missense mutations in the gene for the iron exporter ferroportin. All patients have increased serum ferritin levels, but some have normal transferrin saturation and a mixed pattern of iron accumulation, both in hepatocytes and macrophages. In the typical form (Type 4a, Table 12.2), so-called ferroportin disease, due to loss-of-function ferroportins that are unable to target correctly the cell surface and so export iron from macrophages. The phenotype differs from HH: iron accumulates predominantly in the macrophages (Figure 12.2b) and transferrin saturation is normal or low. In the rare Type 4b with mutations occurring in the binding site of ferroportin for hepcidin, gain-of-function ferroportins reach the cell surface, but are resistant to the effect of hepcidin, causing increased iron export to plasma, saturation of transferrin and iron deposition in hepatocytes similar to *HFE* HH (Figure 12.2a).

Second-level genetic testing is required for the precise diagnosis of non-*HFE* HH. Next-generation sequencing (NGS)-based panels of mutations available in some referral centres may facilitate diagnosis. This approach may also reveal digenic inheritance or mutations in multiple genes. Phlebotomy is indicated as in pC282Y homozygotes; juvenile HH may require even more intensive regimens. Tolerance to phlebotomy should be carefully monitored and intensive regimens are not indicated in ferroportin disease, because of anaemia development. Patients with juvenile forms of HH presenting with life-threatening cardiac failure should be treated with combination of iron chelators (see iron chelation therapy).

Rare genetic causes of iron overload associated with anaemia

Atransferrinaemia

This is a rare recessive disorder associated with a severe hypochromic anaemia with low hepcidin levels and excessive deposition of NTBI in the parenchymal cells. This phenotype underlies the dual role of transferrin to release iron to erythroblasts and to signal for hepcidin synthesis. In all cases tested, some iron-transferrin has been detected by iron-binding ability or immunologically, defining a type of hypotransferrinaemia. Complete absence of transferrin would presumably lead to fetal death. Treatment is based on plasma or transferrin infusions.

DMT1 deficiency

A microcytic hypochromic anaemia with liver iron overload has been described in a few patients with homozygous or doubly heterozygous mutations of *SLC11A2* encoding DMT1. Liver iron stores are increased, but erythroid iron utilisation is impaired and serum hepcidin levels are low for the degree of iron overload. These patients may respond to erythropoietin injections with partial correction of anaemia and iron overload.

Acaeruloplasminaemia

This is a rare recessive disorder in which there is a deficiency of ferroxidase activity as a consequence of mutations in the caeruloplasmin gene (Chapter 11). Clinically, the condition presents in middle age, with progressive degeneration of the retina and basal ganglia and with diabetes mellitus. Iron accumulates in the liver, pancreas and brain, with smaller amounts in the heart, kidneys, thyroid, spleen and retina. Deficiency of ferroxidase activity impairs iron mobilisation from stores and leads to low serum iron and mild hypochromic microcytic anaemia in the presence of normal or high ferritin levels. Early diagnosis requires a high degree of suspicion. Unfortunately, no effective treatment is available to reduce neurodegeneration.

Acaeruloplasminaemia belongs to the group of rare genetic disorders that are classified as neurodegeneration with brain iron accumulation (NIBIA). In these conditions, as well as in Friedereich ataxia and Parkinson disease, iron accumulation may be found in specific areas of the brain, irrespective of systemic iron. Low-dose iron chelation with deferiprone provided limited improvement of iron deposition in prospective double-blind pilot clinical trials of these patients.

Other causes of iron overload

Neonatal haemochromatosis

This is a severe liver disease recognised at birth but usually occurring *in utero*. It is characterised by heavy parenchymal iron deposition in several organs and irreversible liver failure. A genetic cause has been excluded in favour of an *in utero* acquired disorder triggered by an immunological pathogenesis. It is a gestational alloimmune disease due to an alloantibody against fetal hepatocytes, but the target antigen is unknown. The result may be stillbirth or acute liver failure of the newborn or congenital cirrhosis. There is a high risk of recurrence in subsequent pregnancies; thus the disease is familial, but not heritable. Infusions

of γ-globulin in pregnancy appear to reduce the severity of the condition. The other therapeutic option is liver transplantation.

Chronic liver diseases

Moderate iron overload limited to the liver can be found in various chronic viral or metabolic liver diseases in the context of alcohol excess, necro-inflammatory processes and in liver steatosis associated with the so-called metabolic syndrome. Often these conditions are characterised by increased serum ferritin, but normal transferrin saturation and slightly increased hepcidin in the context of an inflammatory process.

'African' iron overload

Historically African iron overload was described in sub-Saharan Africa (Bantu siderosis), as the combination of a dietary component (a traditional beer that contains iron) and a genetic susceptibility unrelated to haemochromatosis genes, but never clearly defined. The proposed variant in ferroportin (pGln248His) that is common in Africa is unlikely to be the cause. Iron deposition is of mixed type and occurs in both hepatocytes and macrophages and may lead to hepatic fibrosis and cirrhosis. Serum ferritin is usually elevated, but transferrin saturation may be normal. In African-Americans iron overload is rare; a common genetic variant (pGlu75del) in the gene that encodes the mechanosensor *PIEZO1*, mutated in inherited stomatocytosis, associates with increased transferrin saturation.

Excess parenteral iron

Erroneous administration of excessive doses of oral iron does not cause iron overload because iron absorption is limited by hepcidin increase. Iron accumulation might occur after high-dose parenteral iron preparations inappropriately administered, due to lack of an iron excretion mechanism. However, the accumulation is usually limited to the reticuloendothelial system.

Hereditary hyperferritinaemia-cataract syndrome

This condition is characterised by isolated hyperferritinaemia not associated with iron overload, early-onset bilateral cataracts and normal serum iron and transferrin saturation. It is due to heterozygous point mutations in the L-ferritin iron-responsive element so that a monoclonal ferritin is synthesised due to impaired negative feedback of ferritin synthesis. The mutation is in the gene promoter and constitutively increases gene transcription. High ferritin accumulates exclusively in the lens, causing congenital or early-onset cataracts.

Iron-loading anaemias

In different forms of anaemia, iron overload occurs when iron intake is increased over a sustained period of time. This may be due either to red blood cell transfusions, as in transfusion-dependent thalassaemia (TDT), or to increased iron absorption through the gastrointestinal tract, because of inhibition of hepcidin synthesis by erythroferrone released from erythroblasts (Chapter 11), as in non-transfusion-dependent thalassaemia (NTDT) and in other anaemias characterised by ineffective erythropoiesis. Secondary iron overload may occur in patients transfused for different forms of inherited and acquired anaemias and in patients after successful allogeneic bone marrow or HSC transplantation for various haematological disorders, who have been heavily transfused.

Iron chelation therapy is essential in patients with transfusion-dependent anaemia, to prevent death from iron overload, usually caused by cardiac failure or arrhythmia. Red blood cell requirements are about 160 mL/kg annually in non-splenectomised and 120 mL/kg annually in splenectomised TDT patients. The iron content of each transfusion is determined by volume (mL) × haematocrit × 1.16 mg. Intake of iron ranges from 0.32 to 0.64 mg/kg body weight daily. Patients with anaemias associated with increased iron absorption who are too anaemic to be venesected to remove iron may also require iron chelation therapy.

The available iron-chelating drugs are: DFO, which is given by slow subcutaneous or intravenous infusion, deferiprone (DFP) and deferasirox (DFX), which are orally active. Their iron-binding properties, pharmacokinetics and routes of elimination differ (Table 12.3).

Iron chelation therapy is monitored by:

1 tests of body iron burden;

2 tests of damage to the organs sensitive to iron overload (Table 12.4);

3 tests to detect potential side-effects of the particular chelating drug being used.

Tests of body iron burden

Serum ferritin

Serum ferritin generally relates to body iron stores and it is relatively inexpensive and easily measured repeatedly. Serum ferritin is useful in monitoring changes in body iron, although measures do not always predict body iron or trends in body iron accurately. This is partly because inflammation increases serum ferritin, while other factors affect it, such as vitamin C status, and partly because the distribution of liver iron between macrophages (Kupffer cells) and hepatocytes has a major impact on plasma ferritin. A sudden increase in serum ferritin should prompt a search for hepatitis, other infections, or inflammatory conditions. The Thalassaemia International Federation (TIF) guidelines recommend maintaining the level below 1000 μg/L in TDT and <800 μg/L in NTDT.

Liver iron

Liver iron may be measured chemically after liver biopsy or by MRI. Chemical estimation was the gold standard, but can be inaccurate if fibrosis is present. Levels >7 mg/g tissue dry weight,

Table 12.3 Characteristics of desferrioxamine, deferiprone and deferasirox.

	Desferrioxamine (DFO)	Deferiprone (DFP)	Deferasirox (DFX)
Structure	Hexadentate	Bidentate	Tridentate
Molecular weight (Da)	560	139	373
Iron–chelator complex	1:1	1:3	1:2
Plasma clearance ($t_{1/2}$)	20 min	1–3 hours	1–16 hours
Absorption	Negligible	Peak 45 min	Peak 1–2.9 hours
Iron excretion	Urine + faecal	Urine	Faecal
Therapeutic daily dose	40 mg/kg	75–100 mg/kg	20–40 mg/kg (dispersible tablet) 7–21 mg/kg (coated tablet)
Route	Parenteral	Oral	Oral
Clinical experience	>40 yrs	>30 yrs	>15 yrs
Side-effects	Ototoxicity, retinal toxicity, growth defects, cartilage and bone abnormalities	Agranulocytosis, arthropathy, gastrointestinal disturbance, transient transaminitis, zinc deficiency	Skin rashes, gastrointestinal disturbance, rising serum creatinine

Table 12.4 Monitoring for iron-induced organ damage.

Cardiac function
ECG ± exercise
24-hour monitoring
Echocardiography, MUGA ± stress test
Doppler echography, MRI

Liver structure and function
Liver function tests
Liver histology
Liver ultrasound
Fibroscan

Bone
Osteoporosis: bone density (DEXA)

Endocrine system
Diabetes: urine glucose, HbA_{1c}, glucose tolerance test, IGF-1
Growth and sexual development: sitting and standing height, Tanner staging, radiography for bone age, testosterone, oestradiol, LH, FSH, SHBG, pulsatile GnRH release, sperm tests
Thyroid: T_4, TSH
Parathyroid: calcium, phosphate, PTH

ECG, electrocardiogram; FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; HbA_{1c}, glycated haemoglobin; IGF, insulin-like growth factor; LH, luteinising hormone; MRI, magnetic resonance imaging; MUGA, multi-gated acquisition scan; PTH, parathyroid hormone; SHBG, sex hormone-binding globulin; TSH, thyroid-stimulating hormone.

and in non-transfusion-dependent anaemia >5 mg/g dry weight are almost always associated with organ damage, while levels <3 mg/g are not associated with clinical complications. Although no clear guidelines are available with liver iron between 3–7 mg/g dry weight, the target of chelation therapy should be to achieve a LIC <3 mg/g dry weight.

MRI techniques are being increasingly used as indirect measures of liver and cardiac iron (Figure 12.4). They have the advantage of being non-invasive and are now widely available. MRI is also the only practical method of performing sequential studies of iron in the liver and heart. Pituitary or other endocrine organs may also be evaluated by MRI, but validation of the software is still required. Different MRI techniques have been used. They all rely on a shortening of relaxation time and thus reduction in signal intensity with iron overload. The R2 or Ferriscan technique is convenient and reproducible for liver iron over the range of clinical interest. The normal ranges of liver iron content by Ferriscan are 0.17–1.8 mg/g dry weight. Gradient echo imaging with the calculation of the $T2^*$ is also applicable, it has a short total imaging time, reducing movement artefacts.

The following formula $[1/(T2^*/1000)] \times 0.0254 + 0.202$ allows us to transform T2* ms in mg/g dw.

Cardiac iron

As cardiac failure or arrhythmia is the usual cause of death in transfusional iron overload, it is essential to monitor cardiac iron. Iron is deposited in myocytes and interstitial fibrosis develops. Direct measurement of cardiac iron by endomyocardial biopsy is inappropriate and inaccurate.

$T2^*$ cardiovascular magnetic resonance offers a reproducible (around 5% coefficient of variation between different observers or between two studies of the same patient), sensitive, albeit indirect measure: the lower the $T2^*$ value, the greater the cardiac iron (Figure 12.5). The majority of patients with $T2^*$ >20 ms have normal left ventricular (LV) function.

A $T2^*$<20 ms correlates with the presence of cardiac dysfunction detected by echocardiography (left and right ventricles) or

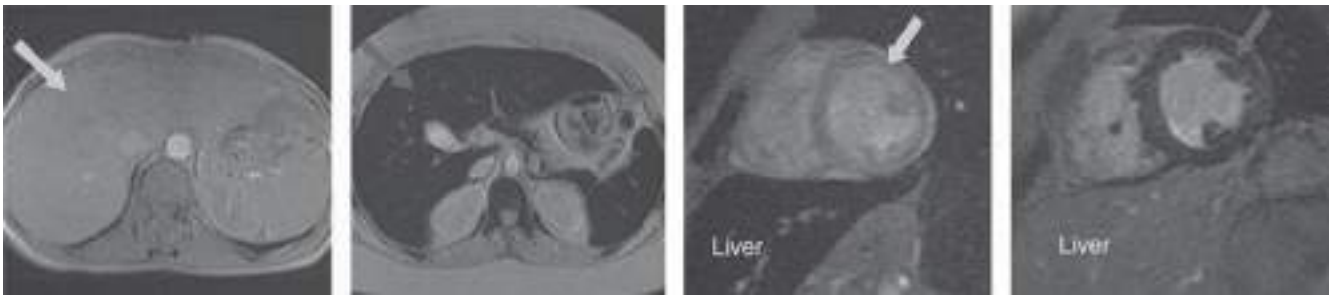


Figure 12.4 Magnetic resonance imaging T2* technique. Tissue appearances of liver and spleen: (a) normal. (b) tissue iron overload. (c) severe liver iron overload with normal cardiac iron. (d) severe cardiac iron deposition with minimal liver iron deposition. White arrows point to normal tissue, grey arrows to iron-loaded tissue (Source (parts (c) and (d)): Anderson *et al.* (2001). Reproduced with permission from Oxford University Press.)

by 24-hour rhythm monitoring or the need for cardiac therapy (Figure 12.5). Most patients who develop cardiac failure have T2* <10 ms. The risk of developing heart failure increases with T2* values <10 ms, which is associated with 160-fold increased risk of heart failure in the next 12 months without adequate chelation. This risk may be substantially less in patients taking regular chelation (see below).

Poor correlation has been found between myocardial iron and liver iron (MRI derived) or serum ferritin in patients receiving iron chelation; thus liver iron and serum ferritin cannot be used as surrogate measures of cardiac iron in these patients.

Urine iron excretion

Iron excretion after a single infusion of a standard dose of DFO or an oral dose of DFP is related to body iron. Urine iron is derived from the labile iron pool chelated mainly extracellularly with DFO and probably intracellularly with DFP. The test may be useful when commencing therapy with DFO or DFP, with which iron excretion is highly dose-related. However, several estimations must be performed at any given dose in view of the variability found.

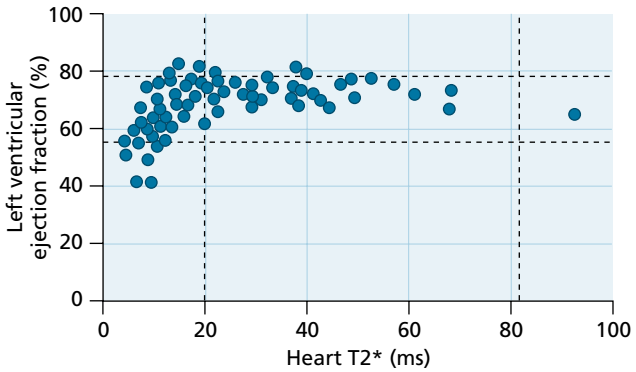


Figure 12.5 Relationships of myocardial T2* and left ventricular ejection fraction in patients with thalassaemia major and iron overload (Source: Anderson *et al.* (2001) *Eur. Heart J.* **22**: 2171–9. Reproduced with permission of Oxford University Press.)

Non-transferrin-bound iron

This is present in the plasma in patients with gross iron overload and high transferrin saturation. It is highly toxic, promoting the formation of free radicals that cause peroxidation of membrane lipids. Part of the early improvement in liver and cardiac function with chelation therapy may be due to removal of this fraction, even before iron burden is substantially lowered. Its clearance by DFO is short-lived as it reappears in plasma within hours of stopping an infusion. DFX, with its long clearance time, provides 24-hour removal of NTBI after a single oral dose. DFP removes NTBI for about 3 hours after a single dose. NTBI is absent from the plasma of well-chelated patients.

Tests for organ damage

The tests that are usually needed are listed in Table 12.4. Heart function is best tested by measurement of left ventricular ejection fraction (LVEF) by echocardiography or by MRI and by tests for rhythm disturbance. Liver function assessment requires routine liver function tests, as well as liver biopsy to assess liver structure, particularly in the presence of chronic viral hepatitis C. When MRI is available to measure the liver iron concentration, abdominal ultrasound and Fibroscan may be used to avoid liver biopsy. α-Fetoprotein should be tested regularly in patients with cirrhosis. The endocrine system is also damaged by iron and appropriate tests are listed in Table 12.4. The anterior pituitary is particularly sensitive, with damage resulting in reduced growth and impaired sexual maturation. Direct damage to the ovaries or testes is usually less important. Hypogonadic hypogonadism, defects of growth hormone secretion and its receptor, and deficiency of insulin-like growth factor mainly account for growth failure. Diabetes mellitus and prediabetes, due to iron deposition in the pancreatic islets, are frequent, especially in patients with genetic susceptibility. Hypothyroidism and hypoparathyroidism are also common in poorly chelated patients. Osteoporosis is well recognised in iron-overloaded thalassaemia patients; it is due to multiple factors and is detected by bone density studies.

Iron chelation therapy

The largest experience in the use of iron chelators comes from patients with thalassaemia and this chapter is prevalently based on such experience. However, since the introduction of oral chelators, especially DFX, iron chelation is increasingly used in several other genetic and acquired anaemias, as outlined below. The main features of the available iron chelating drugs DFO, DFP and DFX are summarised in Table 12.3.

Desferrioxamine

Pharmacokinetics

DFO is not absorbed orally, and after parenteral injection is rapidly cleared from the plasma, being excreted in the urine, taken up by hepatocytes or metabolised in the tissues (Table 12.3). This accounts for the great mobilisation of iron by continuous intravenous or slow subcutaneous infusions, which allow prolonged exposure of the drug to the chelatable iron. It is a trihydroxamic acid (hexadendate), one molecule binding covalently to all six oxygen sites on one ferric ion to form the red chelate, ferrioxamine which is excreted in urine and bile. Faecal iron is derived from hepatocytes. Urinary iron excretion tends to level off at higher doses, but this does not occur with bile excretion so bile iron may predominate at high doses, and this is also the major route of excretion when total body iron has been reduced to relatively low levels.

Clinical studies

DFO was the first chelator used and shown to reduce complications and improve survival in TDT. Its main disadvantage is that it must be administered parenterally, which may often result in poor compliance. The increased toxicity of DFO at low levels of body iron means that guidelines have been conservative, generally recommending not starting therapy until serum ferritin levels reach 1000 ng/mL after approximately 10 units of blood and with care to avoid over-chelation.

In children, tissue damage from iron may be present from very early life; regular iron chelation should begin in TDT after transfusion of about 10 units of blood or when serum ferritin exceeds 1000 µg/L, but it should be started at 20 mg/kg to prevent tissue damage due to iron without causing toxicity due to excess DFO. The standard adult dose of DFO is 40 mg/kg administered subcutaneously given as an 8–12 hour infusion on at least 5 days each week. It is given intravenously at the time of blood transfusion only in patients not complying with any type of chelation and grossly iron overloaded. Vitamin C increases iron excretion by increasing the availability of chelatable iron, but if given in excess may increase the toxicity of iron. It is recommended not to give more than 2–3 mg/kg/day taken at the time of DFO infusion. The response to DFO chelation depends on the amount of blood and consequently of iron being transfused. For those with iron-induced cardiomyopathy, continuous intravenous DFO may be given via an indwelling catheter (e.g. Hickman) or Port-a-Cath chamber. Removal of liver iron is more rapid than removal of cardiac iron with this intensive chelation regimen. Combined therapy with DFP or DFX is indicated if there is iron-induced cardiac disease (see below).

Growth and pubertal development are improved in many, but not all, patients; with adequate chelation diabetes and other endocrine abnormalities are less frequent and MRI studies have shown that about a third of TDT patients chelated with DFO have cardiac $T2^*$ values <10 ms. Through lack of compliance with DFO, premature deaths, usually from iron-induced cardiac damage may occur. Combination therapy or orally active drugs are improving the survival in these DFO failures.

Side-effects

These include rare generalised sensitivity reactions, local soreness related to the site of injection (usually due to the needle being inserted too superficially) and exacerbation of some infections, notably of the urinary tract and precipitation of *Yersinia* enterocolitis. Auditory (high-tone sensorineural hearing loss) and visual neurotoxicity (night blindness, visual field loss, retinal pigmentation) are relatively frequent. Growth and bone defects may also occur. The spine may be affected, with sitting height reduced; rickets-like bone lesions, *genu valgum* and metaphyseal changes are described, especially in children.

Auditory, visual and growth side-effects of DFO occur mainly if the body iron burden is low and doses of DFO high, particularly in children. A therapeutic index can be calculated as follows: mean daily dose (mg/kg)/current serum ferritin (µg/L). If this is below 0.025 at all times, these side-effects of DFO do not occur.

Deferiprone

Pharmacokinetics

Deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one) is an orally rapidly absorbed bidentate, appearing in plasma within 15 min of ingestion (Table 12.3). The chelator–iron complex is excreted with the free drug and glucuronide derivative in urine. Its iron chelation site is inactivated by glucuronidation, the speed of which varies from patient to patient. This explains much of the individual variation in response. It is available both as tablets (500 mg) and as a liquid formulation containing 100 mg/mL, which is particularly useful in children. DFP mobilises iron from parenchymal and reticuloendothelial pools and from transferrin, ferritin and haemosiderin. Its enhanced ability to cross cell membranes may underlie its superior ability compared with DFO to protect the heart from iron. It crosses the blood–brain barrier and has been used in trials to treat neurological conditions with iron loading in the brain.

Clinical studies

The usual dose used has been 75–100 mg/kg daily given as three divided doses. MRI studies suggest that liver iron may generally be higher in patients treated with DFP (75 mg/kg 7 days per week) compared with those treated with DFO (40 mg/kg 5 days per week) or DFX (30 mg/kg daily). In the liver, DFO has the advantage of facilitated transport into cells by an active mechanism. DFP, on the other hand, has greater penetration of myocardial cells. Retrospective and prospective studies have shown, on the basis of $T2^*$ MRI measurement of cardiac iron, echocardiography, clinical incidence of cardiac disease, need for cardiac therapy and survival, that DFP 75 mg/kg is more effective than DFO at protecting the heart from iron-induced cardiomyopathy in routine clinical practice.

Side-effects

The most severe side-effect is agranulocytosis (neutrophils $<0.5 \times 10^9$/L on two consecutive counts), with an estimated incidence of around 0.5–1.0% (0.2–0.3 episodes per 100 patient-years). It is most frequent in the first year of therapy. Lesser degrees of neutropenia ($0.5–1.5 \times 10^9$/L) are more frequent, around 3.5%, occurring more frequently in non-splenectomised patients. Agranulocytosis and neutropenia spontaneously recover when the drug is immediately discontinued, usually within 4–28 days (median duration 9 days), but occasionally are more prolonged. Granulocyte colony-stimulating factor may produce a faster recovery. The mechanism appears to be idiosyncratic, more common in females, with no definite evidence of an immune mechanism established. Patients should be monitored by blood counts every week for at least the first 12 weeks of therapy and every 2 weeks thereafter for 2 years.

Painful joints, especially the knees, occur in around 5–10% in most large series. The incidence has been highest in Indian patients. Some but not all studies show that this complication is most frequent in the most iron-loaded patients and with higher doses of DFP. It usually, but not invariably, resolves with withdrawal of the drug and it is often possible to reintroduce the drug, commencing with lower doses. About 2.0% of patients permanently discontinue therapy because of joint symptoms.

Gastrointestinal side-effects (e.g. nausea and abdominal pain) occur in about 30% of patients in the first year but decrease to 3% in subsequent years. In most, the drug can be reintroduced long-term, initially at a lower dose. The liquid preparation appears to produce fewer GI symptoms. Zinc deficiency has been described in diabetic and prediabetic patients. Rarely, it can lead to clinical features, such as skin rashes and hair loss. It is easily treated by oral zinc therapy. Transient increases in liver enzymes have been associated with DFP therapy in about 7% of patients and about 1% of patients have been withdrawn from therapy because of a persistent rise in liver enzyme levels. Embryo toxicity and teratogenicity have been reported in non-iron-loaded animals treated with deferiprone. Women of childbearing age should be counselled to avoid the drug or use contraception, but a few uneventful pregnancies with healthy newborns have been reported.

Deferasirox

Pharmacokinetics

Deferasirox (DFX), 4-[3,5-bis(2-hydroxyphenyl)-1,2,4-triazol1-yl]benzoic acid, is an oral tridentate chelator forming a 2:1 chelator–iron complex and increases predominantly faecal iron excretion (Table 12.3). It is highly selective for iron. Peak plasma concentration after a single oral dose occurs at about 2 hours, and the drug is still detectable in plasma in almost all patients at 24 hours, with a mean elimination half-life of between 11 and 19 hours after multiple-dose administration. The single daily dose ranges from 20 to 40 mg/kg for dispersible tablets and from 7 to 28 mg/kg for coated tablets. The latter are now the preferred preparation, because, being better tolerated, improves patient compliance.

Clinical studies

DFX has been shown to be effective at eliminating NTBI in plasma and reducing serum ferritin and liver iron in heavily iron-loaded patients. The effect is dependent on dose and on transfusion requirements of the patient. The drug has been shown to be safe and effective in children as young as 2 years. The starting dose in children is 7 mg/kg daily (coated tablet) with subsequent dose adjustments. In adults, 14–28 mg/kg daily of the same preparation may be required, according to iron stores. Fifteen years of experience with DFX in children and adults have shown a tolerable safety profile and there are no reports of DFX having negative impact on growth or sexual development. Available data indicate that DFX is also effective at removing cardiac iron and preventing cardiac siderosis in TDT.

Side-effects

The most common adverse effects have been abdominal pain, nausea, diarrhoea, vomiting and skin rashes. These effects were mainly observed with the dispersible formulation. They decrease in frequency annually and usually respond to dose adjustments, taking the drug in the evening or adding products such as Lactaid to the diet. Non-progressive increases in serum creatinine (defined as a rise above the mean pretreatment measurement by more than 33% on two consecutive tests) occur in about one-third of patients. These increases are dose-dependent and resolve spontaneously. Serum creatinine should be measured in duplicate before therapy and then monthly, with significantly increased levels managed by dose reduction or interruptions.

Combination therapy

Combination therapy of DFO and DFP or DFO and DFX is superior to single-agent therapy. There is evidence for a 'shuttle' effect in which DFP or DFX enters cells, chelates iron and then returns to plasma, where the iron is transferred to DFO for excretion in urine or bile and the oral chelator may re-enter cells. Indications to combination therapy are severely overloaded patients, severe myocardial siderosis and complications of cardiomyopathy especially heart failure.

For DFO and DFP given simultaneously, iron excretion is equivalent to or greater than the use of single agents. Studies of combination therapies, for example, DFP on 7 days a week and DFO on 2 days, have shown a significant fall in serum ferritin and improvement in cardiac and liver iron, improved survival and reversal of endocrinological complications including diabetes, hypothyroidism and hypogonadism. The combination has also proved successful in reversing severe myocardial siderosis. Alternating therapy, for example, 4 or 5 days of DFP and 2 or 3 days of DFO each week, improves compliance and iron status in previously poorly compliant (with DFO) children or adults.

Experience with DFO and DFX combination has been evaluated in a clinical trial (Hyperion) in patients with severe transfusional myocardial siderosis (T2* 5<10 ms, left ventricular ejection fraction (LVEF >56%). The mean dose of DFX was 30.5 mg/kg per day and DFO 36.3 mg/kg per day on a 5-day regimen. A significant improvement of T2* was observed in 24 months and T2* >10 ms was achieved in one-third of the patients, while LVEF was unchanged. Liver iron concentration decreased substantially from a baseline of 33.4–12.8 mg Fe/g dry weight at month 24. The results showed clinically meaningful improvement both in cardiac and liver iron accumulation. Safety was consistent with monotherapies.

Non-transfusion-dependent thalassaemia

Non-transfusion-dependent thalassaemia (NTDT) includes anaemic thalassaemic patients (Hb levels between 70 and 100 g/L) who do not require regular transfusion for survival, as the various genetic forms of β-thalassaemia intermedia, haemoglobin H disease and mild/moderate forms of HbE/ β-thalassaemia (Chapter 15). For these patients, who are not transfusion-dependent or only need a few transfusions each year, iron loading occurs mainly through increased iron absorption due to hepcidin suppression. Venesection is not applicable in most cases because of the severity of the anaemia and danger of increasing bone marrow expansion and bone deformities, thus DFO has been occasionally used in the past. In a randomised clinical trial, DFX has been shown to be effective in 'de-ironing' such patients, reducing serum ferritin and liver iron (Thalassa study). Liver iron concentration should be targeted to below 5 mg/g dry weight to avoid iron toxicity-related complications. It has been shown that in NTDT a serum ferritin level of 300 μg/L corresponds to approximately 3.0 mg LIC, while a serum ferritin level of 800 μg/L corresponds with a level of 5 mg/dry weight.

A rise in haemoglobin level may occur in iron-chelated patients with NTDT. This may be due to removal of iron from the renal oxygen sensor, augmenting the effect of hypoxia and increasing erythropoietin secretion from the kidney. Increased haemoglobin may also be the result of iron restriction that may reduce ineffective erythropoiesis and haemolysis.

Iron chelation in other anaemias

Transfusion-dependent patients with several other inherited (e.g. Diamond–Blackfan syndrome, Fanconi anaemia, sickle cell anaemia and sideroblastic anaemia) or acquired disorders (especially myelodysplasia, myelofibrosis, red cell aplasia or aplastic anaemia) require iron chelation therapy. In these conditions, as well as in elderly patients with acquired, transfusion-dependent, refractory anaemias and otherwise good prognosis, DFO was sporadically used if iron overload was likely to cause significant morbidity or mortality. With the available oral compounds iron chelation in these patients has been implemented with reduction of iron overload and its complications.

Adverse effects in these groups appear similar to those with TDT. The use of DFP in patients with bone marrow failure as in aplastic anaemia and Diamond–Blackfan syndrome should be avoided since agranulocytosis may be more frequent in these patients. As in NTDT, improved haemopoiesis and haemoglobin levels have been described in congenital sideroblastic anaemia and low-risk myelodysplastic syndrome after intensive iron chelation (Chapter 34).

Patients affected by any type of congenital or acquired anaemia cured by bone marrow transplantation may still require iron chelation (in some cases phlebotomy) until safe levels of ferritin are reached.

The increased understanding of the hepcidin function and the results of preclinical studies in animal models indicate that enhancing hepcidin levels might benefit iron-loading anaemias. Based on these results clinical trials are ongoing with new molecules aimed at increasing hepcidin (i.e. inhibitors of *TMPRSS6*) or decreasing iron export to plasma (i.e. inhibitors of ferroportin). The aim is to redistribute iron to the reticuloendothelial system to both decrease toxicity and reduce ineffective erythropoiesis.

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CHAPTER 13

Megaloblastic anaemia

13

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Introduction

The megaloblastic anaemias are a group of disorders characterised by the distinctive morphological appearances of developing erythroid cells in the bone marrow. The cause is usually deficiency of either cobalamin (vitamin B_{12}, B12) or folate, but megaloblastic anaemia may arise because of inherited or acquired abnormalities affecting the metabolism of these vitamins or because of defects in DNA synthesis not related to cobalamin or folate (Table 13.1).

Underlying basic science

Biochemical basis of megaloblastic anaemia

The common feature of all megaloblastic anaemias is a defect in DNA synthesis that affects rapidly dividing cells of the bone marrow and of other tissues. All conditions that give rise to megaloblastic changes share in common a reduced rate of synthesis or of polymerisation of the four immediate precursors of DNA: the deoxyribonucleoside triphosphates (Figure 13.1). In deficiencies of either folate or cobalamin, there is a failure to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). The coenzyme 5,10-methylene tetrahydrofolate polyglutamate is needed for this reaction and the availability of this co-enzyme is reduced in either deficiency (see further on).

The reduced supply of deoxythymidine triphosphate (dTTP) in megaloblastic anaemia due to folate or cobalamin deficiency slows elongation and ligation of newly originated replicating segments of DNA at multiple sites of origin during cell mitosis. Thus, small DNA fragments accumulate, single-stranded areas become points of weakness where mechanical or enzymatic breakage may occur, and the failure to form bulk DNA impairs contraction of newly replicated lengths of DNA, leaving the chromosomes elongated, despirillated and with random breaks. Late-replicating DNA is particularly affected and some cells become arrested at this stage and die by apoptosis. An alternative hypothesis for megaloblastic anaemia in cobalamin or folate deficiency is the misincorporation of uracil into DNA because of a build-up of deoxyuridine triphosphate (dUTP) at the replication fork as a consequence of the block in conversion of dUMP to dTMP (Figure 13.1). Data on this hypothesis are conflicting. It does not explain megaloblastic anaemia due to defects of DNA synthesis at sites other than at thymidylate synthesis, for example with drugs such as hydroxycarbamide (hydroxyurea), cytarabine or 6-mercaptopurine, or with enzyme deficiencies such as orotic aciduria or thiamine-responsive megaloblastic anaemia (see further on).

Cobalamin–folate relationship

Folate is required for many other reactions in mammalian tissues, including two in purine synthesis (Table 13.2), but impairment of these is far less important clinically. Only two reactions in the body are known to require cobalamin (Figure 13.2). Methylmalonyl-CoA isomerisation, which requires deoxyadenosyl(ado)-cobalamin, is discussed later.

The methylation of homocysteine to methionine requires both 5-methyltetrahydrofolate (methyl-THF) as methyl donor and

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 13.1 Causes of megaloblastic anaemia.

Cobalamin deficiency or abnormalities of cobalamin metabolism (Table 13.4)
Folate deficiency or abnormalities of folate metabolism (Table 13.6)
Therapy with antifolate drugs (e.g. methotrexate)
Independent of either cobalamin or folate deficiency and refractory to cobalamin and folate therapy:
Some cases of acute myeloid leukaemia, myelodysplasia*
Therapy with drugs interfering with DNA synthesis (e.g. cytarabine, hydroxycarbamide, 6-mercaptopurine, azidothymidine)
Orotic aciduria (responds to uridine)
Lesch–Nyhan syndrome (may respond to adenine)

* Folate deficiency also occurs frequently in these diseases.

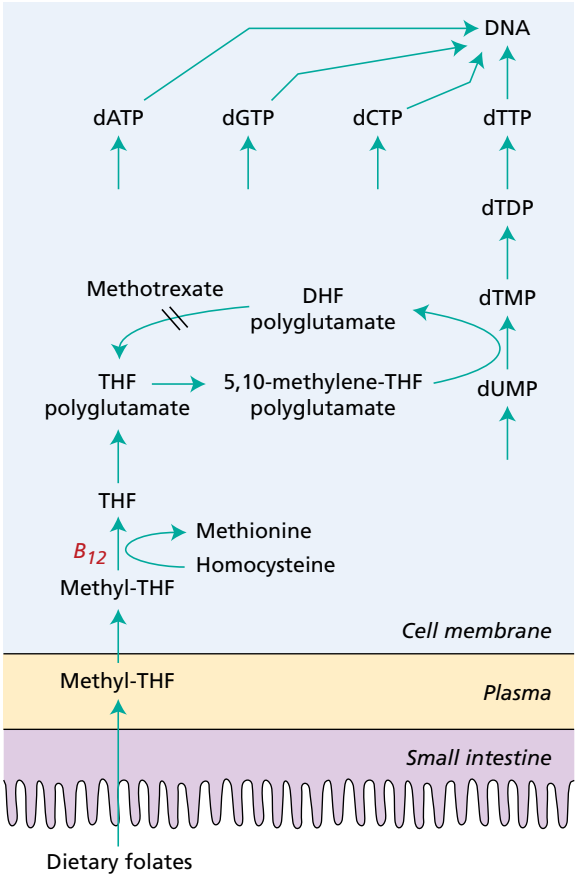


Figure 13.1 Role of folate (as 5,10-methylene-THF polyglutamate) and methylcobalamin in DNA synthesis. A, adenine; B12, vitamin B12, cobalamin; C, cytosine; d, deoxyribose; G, guanine; MP, monophosphate; T, thymine; THF, tetrahydrofolate; TP, triphosphate.

methylcobalamin as co-enzyme (Figures 13.1 and 13.3). This reaction, which is almost completely irreversible, is the first step in the pathway by which methyl-THF, which enters bone marrow and other cells from plasma, is converted into all the intracellular folate co-enzymes (Figure 13.1). The co-enzymes are all polyglutamated (the larger size aiding retention in the cell), but the enzyme folate polyglutamate synthase requires THF and not methyl-THF as substrate. In cobalamin deficiency, methyl-THF accumulates in the plasma, while intracellular folate concentrations fall due to failure of formation of intracellular folate polyglutamates including 5,10-methylene-THF because of 'THF starvation'. The 'methylfolate trap' will also cause THF starvation at the polyglutamate level.

This theory explains the abnormalities of folate metabolism that occur in cobalamin deficiency (high serum folate, low cell folate, reduced thymidylate synthesis, raised purine precursor AICAR excretion; Table 13.2) and also why the anaemia that occurs in cobalamin deficiency will respond to folic acid in large doses. The explanation of why serum cobalamin falls in folate deficiency may also be related to impairment of the homocysteine–methionine reaction, with reduced formation of methylcobalamin, the main form of cobalamin in plasma, but other mechanisms may be responsible.

Clinical features

Many symptomless patients are detected through the finding of a raised mean corpuscular volume (MCV) on a routine blood count. The main clinical features in more severe cases are those of anaemia. Anorexia is usually marked and there may be weight loss, diarrhoea or constipation. Other particular features include glossitis, angular cheilosis, a mild fever in the more severely anaemic patients, jaundice (unconjugated) and reversible melanin skin hyperpigmentation. Thrombocytopenia sometimes leads to bruising (and this may be aggravated by vitamin C deficiency in malnourished patients). The low leucocyte count may predispose to infections, particularly of the respiratory or urinary tracts. Cobalamin deficiency has also been associated with impaired bactericidal function of phagocytes.

General tissue effects of cobalamin and folate deficiencies

Epithelial surfaces

These deficiencies, when severe, affect all rapidly growing (DNA-synthesising) tissues. After the marrow, the next most affected tissues are the epithelial cell surfaces of the mouth, stomach, small intestine and respiratory, urinary and female genital tracts. The cells show macrocytosis, with increased numbers of multinucleate and dying cells. The deficiencies may cause cervical smear abnormalities.

Complications of pregnancy

The gonads are also affected and infertility is common in both men and women with either deficiency if severe. Maternal folate deficiency has been implicated as a cause of prematurity and both folate and cobalamin deficiency have been implicated in recurrent fetal loss.

Table 13.2 Biochemical reactions of folate co-enzymes.

Reaction	Co-enzyme form of folate involved	Single-carbon unit transferred	Importance
Formate activation	THF	—CHO	Generation of 10-formyl-THF
Purine synthesis Formation of glycinamide ribonucleotide	5,10-Methenyl-THF	—CHO	Formation of purines needed for DNA, RNA synthesis, but reactions probably not rate limiting
Formylation of aminoimidazole-carboxamideribotide (AICAR)	10-Formyl-THF	—CHO	
Pyrimidine synthesis Methylation of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP)	5,10-Methenyl-THF	$—CH_3$	Rate limiting in DNA synthesis Oxidises THF to DHF Some breakdown of folate at the C-9–N-10 bond
Amino acid interconversion Serine–glycine interconversion	THF	$=CH_2$	Entry of single-carbon units into active pool
Homocysteine to methionine	5-Methyl-THF	$—CH_3$	Demethylation of 5-methyl-THF to THF; also requires cobalamin, flavine adenine dinucleotide, ATP and adenosylmethionine
Forminoglutamic acid to glutamic acid in histidine catabolism	THF	—HN—CH=	Basis of the Figlu test (now obsolete)

DHF, dihydrofolate; THF, tetrahydrofolate.

Neural tube defects

Folic acid supplements at the time of conception and in the first 12 weeks of pregnancy when the neural tube is forming reduce, if 5 mg daily, by 80% the incidence of neural tube defects (NTDs) – anencephaly, meningomyelocele, encephalocele and spina bifida – in the fetus. Most of this protective effect can be achieved by taking folic acid 0.4 mg daily. The incidence of cleft palate and harelip can also be reduced by prophylactic folic acid. There is no clear simple relationship between maternal folate status and these fetal abnormalities, although the lower the maternal folate (and cobalamin) plasma levels, the greater the risk of NTD or, in diabetic mothers of other birth defects, in

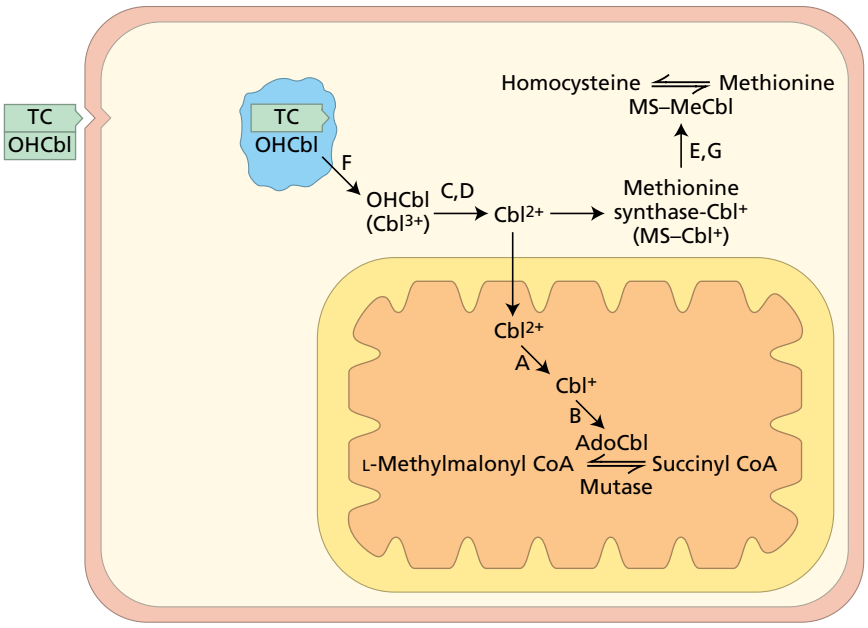


Figure 13.2 Intracellular cobalamin metabolism. Cbl^{1+}, Cbl^{2+} and Cbl^{3+} refer to the oxidation state of the central cobalt atom of cobalamin Cbl^{3+} being the most oxidised. A–G refers to the sites of blocks that have been identified by complementation analysis in infants with metabolic defects. AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; TC, transcobalamin. The mitochondrial, lysosomal and cytoplasmic compartments are indicated (Source: Lilleyman JS, Hann IM, and Blanchette VS (eds) (1999) *Paediatric Haematology*, 2nd edn. Churchill Livingstone, Edinburgh. Reproduced with permission from Elsevier.)

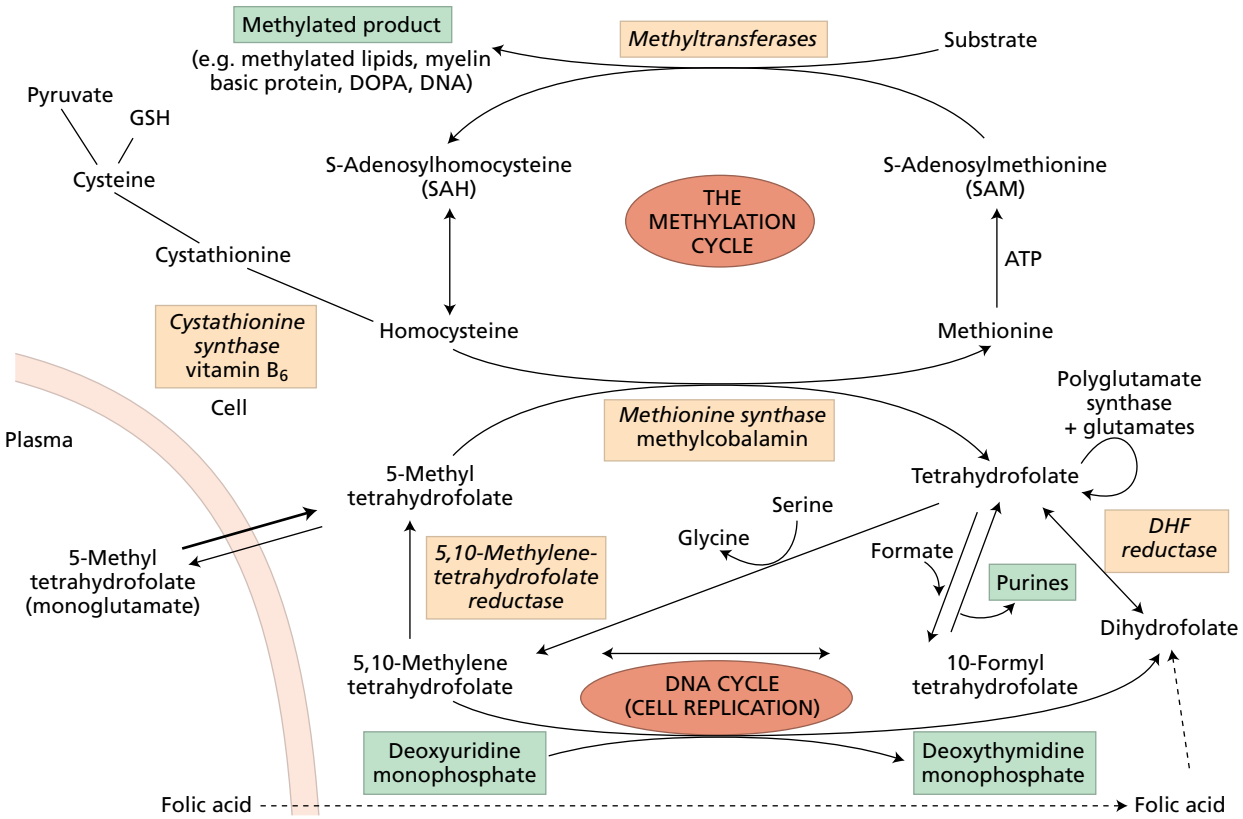


Figure 13.3 The role of folates in DNA synthesis and in formation of *S*-adenosylmethionine (SAM), which is involved in numerous methylation reactions. Enzymes are shown in pink boxes. (Figure prepared in conjunction with Professor John Scott.)

infants. Doubling the maternal serum folate level approximately halves the risk of an NTD pregnancy. This applies even when the maternal serum folate level is in the conventional normal range. NTDs can also be caused by antifolate and antiepileptic drugs, especially valproic acid. There is also an association between lower maternal cobalamin levels and an increased incidence of an NTD pregnancy.

An underlying maternal folate metabolic abnormality has also been postulated. One abnormality has been identified: reduced activity of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) (Figure 13.3) caused by a common 677C→T polymorphism in the *MTHFR* gene. In one study, the prevalence of this polymorphism was found to be higher in the parents of NTD fetuses and in the fetuses themselves: homozygosity for the TT mutation was found in 13% compared with 5% in control subjects. The polymorphism codes for a thermolabile form of MTHFR. The homozygous state results in lower mean serum and red cell folate compared with control subjects, as well as significantly higher serum homocysteine levels. Tests for mutations in other enzymes possibly associated with NTDs, have been negative.

About 20% of NTDs cannot be prevented even by high doses of folic acid. It seems that metabolic abnormalities unrelated to folate underlie these.

Cardiovascular disease

Children with severe homocystinuria (blood levels of 100 µmol/L or more) due to deficiency of one of three enzymes, methionine synthase, MTHFR or cystathionine synthase (Figure 13.3) suffer from vascular disease (e.g. ischaemic heart disease, cerebrovascular disease or pulmonary embolus) as teenagers or in young adulthood. Meta-analysis shows a significant association between lesser degrees of raised serum homocysteine (normal range 5–15 µmol/L) and of homozygosity for mutated *MTHFR* with ischaemic heart disease, stroke, deep vein thrombosis and pulmonary embolism.

It remains possible that homocysteine levels may be high as a consequence of vascular damage or may merely be a marker for some other underlying factor that is responsible for both the vascular damage and the raised homocysteine. Folate deficiency, for example, may be such a factor. Folate levels have been found in various studies to be lower in patients with myocardial infarct and carotid artery disease than control subjects. Meta-analysis of data from large multicentre prospective trials of folic acid in prevention of coronary arterial disease, however, does not show a positive effect, whereas a reduction of the risk of stroke by 18% has been reported from China, but even for this, there are conflicting data. For coronary disease aspirin may over-ride any effect of folic acid.

Extremely high levels of homocysteine (>50 μmol/L) are toxic to endothelia. When homocysteine levels are only mildly or moderately elevated, other mechanisms have been proposed, including oxidant damage interaction of homocysteine with cysteine residues on coagulation factors, platelets, adhesion molecules or endothelial cells or promotion of vascular wall inflammation.

Malignancy

Prophylactic folic acid in pregnancy has been found in some, but not all, studies to reduce the subsequent incidence of acute lymphoblastic leukaemia (ALL) in childhood. A significant negative association has been found with the *MTHFR* 677C→T and 1298A→C polymorphisms and the incidence of both paediatric and adult ALL. There are various positive and negative associations between polymorphisms in other folate-dependent enzymes and the incidence of paediatric and adult ALL. Other tumours that have been associated with folate polymorphisms or status include follicular lymphoma, breast cancer and gastric cancer.

Meta-analysis of 10 randomised trials of folic acid at daily doses of 0.5 mg or more for an average of 5.2 years to prevent coronary heart disease, as well as of three trials for prevention of colonic adenomas (total 49,621 individuals) has not shown a significant difference for cancer generally or for any specific cancer between folic acid and placebo. This is reassuring, both for those taking folic acid as a vitamin supplement and for countries where food fortification with folic acid is carried out.

Other tissues

Folate deficiency causes reduced regeneration of cirrhotic liver. Patients with gluten-induced enteropathy and those with sickle cell anaemia have also been reported to show stunted growth, which has been improved coincidentally with commencement of folic acid therapy, but it is not certain how much the growth improvement in these children was due to folic acid and how much to other, simultaneously administered vitamins. There are experimental data in animals and some clinical observations that long-term cobalamin deficiency can affect bone formation. In the fragile X syndrome, sister chromatid exchange and DNA breaks are increased *in vitro* in a folate-deficient medium, apparently at the Xq28 site. No *in vivo* abnormality of folate metabolism can be detected.

Neurological manifestations

Cobalamin deficiency may cause bilateral peripheral neuropathy or degeneration (demyelination) of the posterior and pyramidal tracts of the spinal cord and, less frequently, optic atrophy or cerebral symptoms. The patient classically presents with paraesthesiae in the legs, muscle weakness or difficulty in walking and sometimes dementia, psychotic disturbances or visual impairment. Orthostatic hypotension has also been described as a complication of even mild cobalamin deficiency and in a few small studies the underlying autonomic neuropathy shown to improve with cobalamin therapy. Long-term nutritional cobalamin deficiency in infancy leads to poor brain development and impaired intellectual development. Folate deficiency may cause mental changes such as depression and slowness, and has been suggested to cause organic nervous disease, but this is uncertain. Methotrexate injected into the cerebrospinal fluid may cause brain or spinal cord damage. Neural tube defects in the fetus are discussed above.

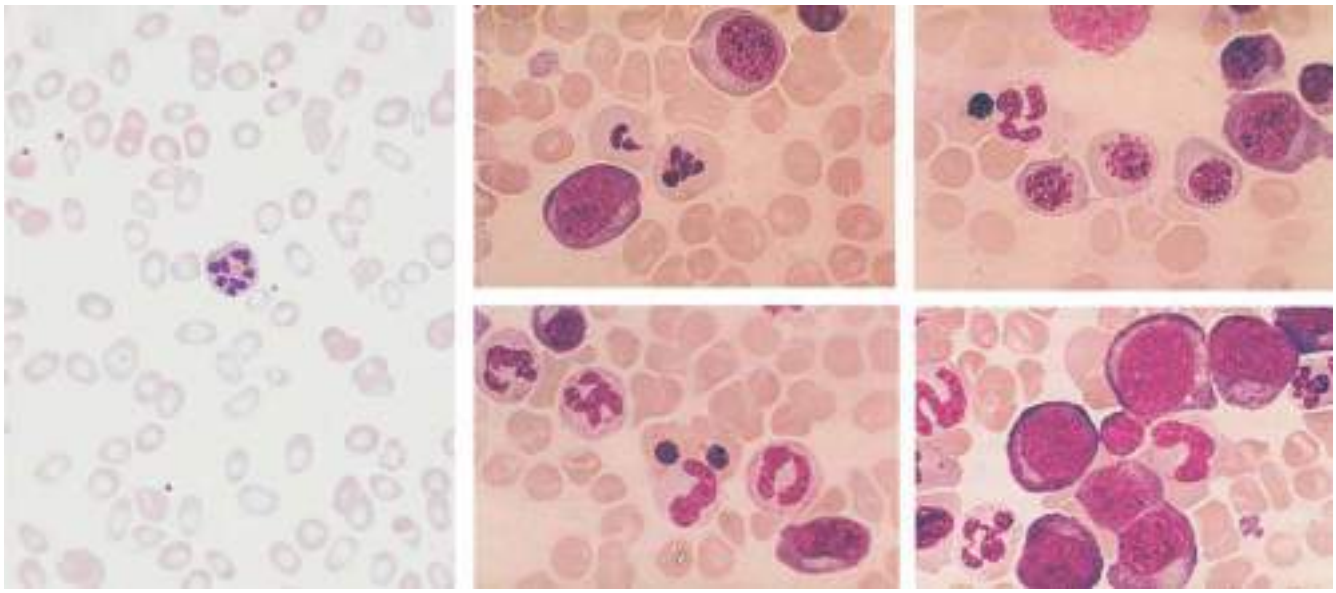
Studies showing an association between lower serum levels of folate or cobalamin and higher homocysteine levels and Alzheimer disease, loss of cognitive function or brain volume loss have been reported. Serum cobalamin levels even in the low normal range if associated with raised homocysteine levels have also been associated with cognitive impairment. One trial has shown that supplementation with folic acid, cobalamin and vitamin B_6 delayed brain atrophy in such subjects with also raised serum homocysteine levels compared to a control group. More recent trials mainly in China have shown a benefit of cobalamin and folic acid in improving or slowing progression of mild cognitive impairment but such benefit remains controversial.

The biochemical basis for cobalamin neuropathy remains obscure. Its occurrence in the absence of methylmalonic aciduria in transcobalamin deficiency, and monkeys given nitrous oxide (N_2O), suggests that the neuropathy is related to the defect in conversion of homocysteine to methionine. Accumulation of *S*-adenosylhomocysteine (SAH) in the brain, resulting in inhibition of transmethylation reactions due to an altered *S*-adenosylmethionine (SAM) to SAH ratio, has been suggested. SAM is needed in methylation of biogenic amines (e.g. dopamine), as well as of proteins, phospholipids and neurotransmitters in the brain (Figure 13.3).

Haematological findings

Peripheral blood

Oval macrocytes, usually with considerable anisocytosis and poikilocytosis, are the main features (Figure 13.4a). The MCV is usually more than 100 fL unless a cause of microcytosis (e.g. iron deficiency or thalassaemia trait) is present when there is a raised red cell distribution width (RDW) and the film is dimorphic. The MCV may also be normal owing to excess fragmentation of red cells. Some of the neutrophils are hypersegmented (more than five nuclear lobes). Both macrocytosis and hypersegmented neutrophils may also occur in other situations (Table 13.3). Together, however, they strongly suggest megaloblastic haemopoiesis. There may be leucopenia due to a reduction in granulocytes and lymphocytes; the platelet count may be moderately reduced, rarely to less than 40×10^9/L.



(a) (b)

Figure 13.4 Severe megaloblastic anaemia: (a) peripheral blood (Source: Historical Library.) (b) bone marrow.

Occasionally, a leucoerythroblastic blood picture is seen. In the non-anaemic patient, the presence of a few macrocytes and hypersegmented neutrophils in the peripheral blood may be the only abnormalities.

Table 13.3 Conditions in which macrocytosis or hypersegmented neutrophils may occur in the absence of megaloblastic anaemia.

Macrocytosis
Alcohol
Liver disease (especially alcoholic)
Reticulocytosis (haemolysis or haemorrhage)
Aplastic anaemia or red cell aplasia
Hypothyroidism
Myelodysplasia
Myeloma and macroglobulinaemia
Leucoerythroblastic anaemia
Myeloproliferative disease
Pregnancy
Newborn
Congenital dyserythropoietic anaemia (type II)
? Chronic respiratory failure
Hypersegmented neutrophils
Renal failure
Congenital (familial)
? Iron deficiency

Falsely high MCV recorded when cold agglutinins, paraproteins or marked leucocytosis are present.

Bone marrow

In severely anaemic patients, the marrow is hypercellular with accumulation of primitive cells due to selective death of more mature forms. There is dissociation between nuclear and cytoplasmic development in the erythroblasts, with the nucleus maintaining a primitive appearance despite maturation and haemoglobinisation of the cytoplasm; fully haemoglobinised (orthochromatic) erythroblasts may be seen. The nucleus of the megaloblast has an open, fine and lacy appearance; the cells are larger than normoblasts and an increased number of cells with eccentric lobulated nuclei or nuclear fragments may be present (Figure 13.4b). Mitotic and dying cells are seen more frequently than normal. Giant and abnormally shaped metamyelocytes and enlarged hyperpolyploid megakaryocytes are characteristic. Severe florid megaloblastic changes may be confused with acute erythroid leukaemia. Rarely, the marrow may be hypocellular or red cell precursors are lost almost completely from the marrow and a mistaken diagnosis of myeloid leukaemia may be made. Iron staining shows increase in both reticuloendothelial stores and in the developing megaloblasts.

In less anaemic patients, the changes in the marrow may be difficult to recognise. The terms 'intermediate', 'mild' and 'early' have been used. The changes may be mild and difficult to recognise, even in a severely anaemic patient, if the anaemia is largely due to other factors (e.g. iron deficiency, infection, malignant disease and haemolysis) and the megaloblastosis is an incidental phenomenon. The term 'megaloblastoid' has several different connotations, including the dysplastic changes seen in the myelodysplastic syndromes, and is best avoided.

Chromosomes

Bone marrow cells, transformed lymphocytes and other proliferating cells in the body show a variety of changes including random chromosomal breaks, reduced contraction of chromatin, spreading of the centromere, and exaggeration of secondary chromosomal constrictions and prominent satellites. Similar abnormalities may be produced by antimetabolite drugs (e.g. cytarabine, hydroxycarbamide and methotrexate) that interfere with either DNA replication or folate metabolism and which also cause megaloblastic appearances.

Ineffective haemopoiesis

There is accumulation of unconjugated bilirubin in plasma due to the death of nucleated red cells in the marrow (ineffective erythropoiesis). Other evidence for this includes raised urine urobilinogen, reduced haptoglobins and positive urine haemosiderin, raised serum lactate dehydrogenase and raised serum iron, non-transferrin-bound iron and ferritin levels. Carbon monoxide production has also increased. Serum lysozyme may also be raised, suggesting ineffective granulopoiesis.

In rare patients, ineffective haematopoiesis is associated with features of disseminated intravascular coagulation, with raised serum fibrin degradation products. Thrombocytopenia, when it occurs, is usually caused by ineffective megakaryopoiesis. A weakly positive direct antiglobulin test due to complement can lead to a false diagnosis of autoimmune haemolytic anaemia.

Cobalamin

Cobalamin (vitamin B_{12}) exists in a number of different chemical forms. The molecule consists of two halves: a planar group and a nucleotide set at right angles to it (Figure 13.5). The planar group is a corrin ring and the nucleotide consists of a base, 5,6-dimethylbenzimidazole, and a phosphorylated sugar, ribose-5-phosphate. In nature, the vitamin is mainly in the 5′-deoxyadenosyl (ado) form. This is the main form in human tissues and is located in the mitochondria. It serves as the co-factor for methylmalonyl-CoA mutase. The other major natural cobalamin is methylcobalamin, the main form in human plasma and cell cytoplasm, which serves as the co-factor for methionine synthase. There are also minor amounts of hydroxocobalamin, the form to which methyl- and adocobalamin are rapidly converted by exposure to light, hydroxocobalamin having its cobalt atom in the fully oxidised Cbl^{3+} state, whereas the cobalt exists as reduced Cbl^{1+} in the methyl- and ado-cobalamin forms (see Figure 13.2).

Dietary sources and requirements

Cobalamin is synthesised solely by microorganisms. Ruminants obtain cobalamin from the foregut, but the only source for humans is food of animal origin. The highest amounts are found

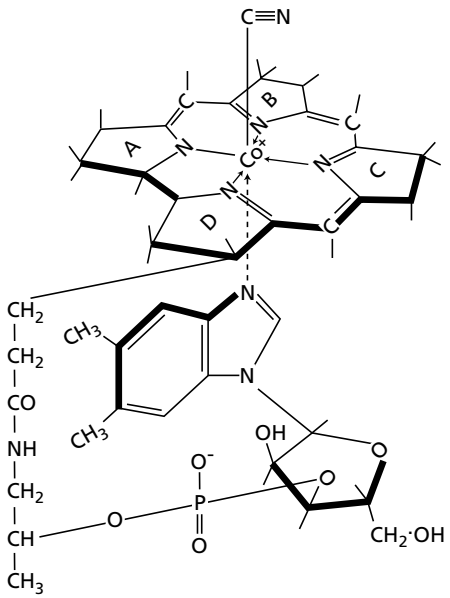


Figure 13.5 The structure of vitamin B_{12} (cyanocobalamin). There is a corrin ring with a cobalt atom at its centre. Above is attached the cyano, hydroxo, methyl or deoxyadenosyl ligand. Below is the nucleotide 5,6-dimethylbenzimidazole.

in liver and kidney (up to 100 µg per 100 g), but it is also present in shellfish, organ and muscle meats, fish, chicken and dairy products (eggs, cheese and milk) in small amounts (6 µg/L). Vegetables, fruits and all other foods of non-animal origin are free from cobalamin unless they are contaminated by bacteria. Cooking does not usually destroy cobalamin.

A normal Western diet contains 5–30 µg of cobalamin daily. Adult daily losses (mainly in the urine and faeces) are about 1–2 µg and because the body does not have the ability to degrade cobalamin, daily requirements are also about 1 µg. Body stores are of the order of 2–3 mg and are sufficient for 3–4 years if supplies are completely cut off.

Absorption

Two mechanisms exist for cobalamin absorption. One is passive, occurring equally through the duodenum and the ileum; it is rapid but inefficient as less than 1% of an oral dose can be absorbed by this process. Passive absorption of cobalamin can also occur through other mucous membranes such as the sublingual and nasal mucosae. The other mechanism is active; it occurs through the ileum in humans and is efficient for small (a few micrograms) oral doses of cobalamin. This is the normal mechanism by which the body acquires cobalamin and is mediated by gastric intrinsic factor (IF).

Dietary cobalamin is released from protein complexes by enzymes in the stomach, duodenum and jejunum; it combines rapidly with a salivary gastric glycoprotein (R binder) related to

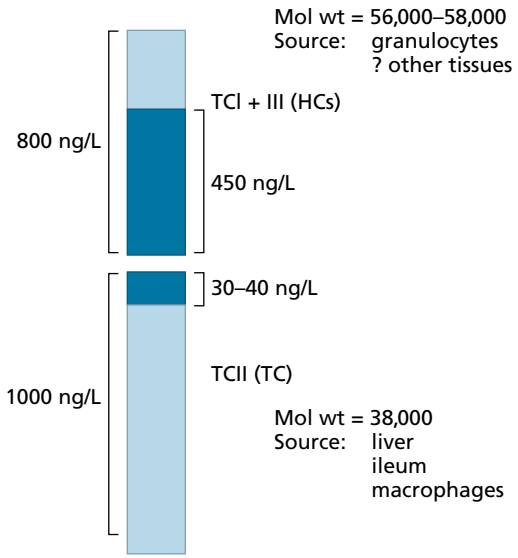


Figure 13.6 The serum cobalamin-binding proteins (TCs, transcobalamins). Dark blue rectangles indicate endogenous serum cobalamin; light blue rectangles indicate unsaturated cobalamin-binding protein; HCs, haptocorrins.

plasma transcobalamin I (TCI). These proteins are isoforms of cobalamin-binding proteins known as haptocorrins (HCs), which differ from each other only in their patterns of glycosylation. They are products of a single gene (*TCN1*), and they occur in saliva, gastric juice, bile, milk and other body fluids. Subsequently, HC is digested by pancreatic trypsin and the cobalamin transferred to IF. Binding of cobalamin to IF is favoured by an alkaline pH; it binds one molecule for one molecule. All forms of cobalamin are absorbed by the same IF mechanism (Figure 13.6). Intrinsic factor is a glycoprotein (molecular weight 45,000) encoded by a gene on chromosome 11q13. It is produced in gastric parietal cells in the fundus and body of the stomach. The IF–cobalamin complex, in contrast with free IF, is resistant to enzyme digestion, having a more closed structure. It passes to the ileum, where IF attaches to a specific receptor (cubilin) on the brush border surface of the ileal absorptive cells. Cubilin is also present in the yolk sac and renal proximal tubular epithelium. The attachment of the IF–cobalamin complex requires calcium ions and a pH around neutral. It is probably a physical process, not requiring energy. Cubilin traffics by means of the protein called amnionless (AMN). AMN binds tightly to cubilin and directs sublocalisation and endocytosis of cubilin with its ligand (IF–cobalamin complex). Defects in cubilin and AMN are implicated in autosomal recessive megaloblastic anaemia, characterised by intestinal malabsorption of cobalamin. A third protein, megalin (LRP2), has been suggested to play a role in stabilising the cubilin–AMN complex.

Cobalamin then enters the ileal cell. IF is digested and after a delay of about 6 hours absorbed cobalamin appears in portal blood attached to transcobalamin (TCII), which is synthesised in the ileum, either by mucosal cells or by venous endothelial cells in the submucosa.

The ileum has a restricted capacity to absorb cobalamin because of limited receptor sites. Although 50% or more of a single dose of 1 µg of cobalamin may be absorbed, with doses above 2 µg the proportion absorbed falls rapidly. Moreover, after one dose of IF–cobalamin complex has been presented, the ileal cells become refractory to further doses for about 6 hours.

Enterohepatic circulation

Between 0.5 and 5.0 µg of cobalamin enter the bile each day. This binds to IF and a portion of biliary cobalamin is reabsorbed together with cobalamin derived from sloughed intestinal cells. Bile may enhance cobalamin absorption. Cobalamin deficiency develops more rapidly in individuals who malabsorb cobalamin than it does in vegans, who ingest no cobalamin but in whom reabsorption of biliary cobalamin is intact.

Transport

Two main cobalamin-binding proteins exist in human plasma; they both bind cobalamin one molecule for one molecule (Figure 13.6). One HC (also known as TCI) is a glycoprotein. TCIII is a minor isoprotein of TCI in plasma. These HCs are derived primarily from the specific granules in neutrophils and are normally about two-thirds saturated with cobalamin, which they bind tightly. They do not enhance cobalamin entry into tissues. Glycoprotein receptors on liver cells are concerned with the removal of HCs from plasma, and HC may have a role in the transport of cobalamin analogues to the liver for excretion in bile. The gene, *TCN1* on chromosome 11q11–q12.3, has nine exons and codes a protein of 433 amino acids. Heterozygous, homozygous or compound heterozygous mutations in the gene may cause mild or severe reductions in serum vitamin B_{12}, respectively, with no known clinical consequences.

The other major cobalamin transport protein in plasma is transcobalamin (TC, also known as TCII). This is synthesised by liver, and by other tissues, including macrophages, endothelial and possibly ileal cells. It normally carries only 20–60 ng of cobalamin per litre of plasma and readily gives up cobalamin to marrow, placenta and other tissues, which it enters by receptor-mediated endocytosis via clathrin-coated pits. TC is not reutilised. The gene is on chromosome 22q11–q13.1 and, as for IF and HC, there are nine exons. The three proteins are likely to have a common ancestral origin. TC has 20% amino acid homology and more than 50% nucleotide homology with human HC and with rat IF. The regions of homology of HC, TC and IF are involved in cobalamin

binding. Five different inherited isoproteins of TC have been described; all are functionally active. TC is also present in cerebrospinal fluid and binds cobalamin (approximately 10 ng/L) there. Alterations may occur in TC and HC levels in a variety of disease states. In general, an increase in HC is found particularly in myeloproliferative diseases and hepatoma and causes an increase in serum cobalamin, whereas an increase in TC does not.

The TC receptor belongs to the low-density lipoprotein receptor family. It is more heavily expressed in dividing than quiescent cells and is recycled to the cell surface. Megalin (LRP-2) is also involved in the endocytosis of TC–cobalamin.

Cobalamin analogues

Cobalamin analogues are corrinoids, which exist as cobamides (containing substitutions in the place of ribose, e.g. adenosyl) or as cobinamides (which have no nucleotide whatever). They do not bind to IF and are not absorbed. HC may carry analogues to the liver for excretion in the bile. It is unclear whether they are inert or inhibit cobalamin-dependent reactions. The proportion of analogues derived from diet, gut bacteria or endogenous breakdown of cobalamins is unknown. They are present in fetal blood and tissues.

Causes of cobalamin deficiency

Cobalamin deficiency is usually due to malabsorption. The only other cause is inadequate dietary intake. Cobalamin deficiency due to excess degradation does not occur. Inactivation may occur as a result of exposure to the anaesthetic gas N_2O. N_2O causes irreversible oxidation of the active Cbl^{1+} during catalytic shunting of labile methyl groups in the methionine synthase reaction (see Figure 13.2).

Inadequate dietary intake adults

Dietary cobalamin deficiency arises in vegans who omit meat, fish, eggs, cheese and other animal products from their diet. The largest group in the world consists of Hindus, and many millions of Indians are at risk of deficiency on a nutritional basis. However, not all vegans develop cobalamin deficiency of sufficient severity to cause anaemia or neuropathy, even though subnormal cobalamin levels have been found in up to 50% of randomly selected, young, adult Indian vegans. Dietary cobalamin deficiency may also arise rarely in non-vegetarian subjects who exist on grossly inadequate diets because of poverty or psychiatric disturbance. Nutritional cobalamin deficiency may not progress to megaloblastic anaemia because the diet is not totally lacking cobalamin, the enterohepatic circulation is intact and body losses will reduce as stores diminish.

Infants

Cobalamin deficiency occurs in infants born to severely cobalamin-deficient mothers. These infants develop megaloblastic anaemia at about 3–6 months of age since they are born with low stores of cobalamin and are then fed breast milk of low cobalamin content. This occurs most commonly not only in Indian vegans, but also in unrecognised maternal pernicious anaemia and in strict practitioners of veganism living in Western countries whose offspring have shown growth retardation, impaired psychomotor development and other neurological sequelae.

Gastric causes of cobalamin malabsorption

Pernicious anaemia

Pernicious anaemia (PA) may be defined as a severe lack of IF due to gastric atrophy (Tables 13.4 and 13.5). It is a common disease in northern Europeans but occurs in all countries and ethnic groups. The overall incidence is about 120 per 100,000 population in the UK, but there is wide variation between one area and the next. The prevalence rate in Western countries may be as high as 2–3%. The ratio of incidence in men and women is approximately 1:1.6 and the peak age of onset is 60 years, with only 10% of patients being less than 40 years of age. In some ethnic groups, notably black people and Latin Americans, the age of onset of PA is generally lower. The disease occurs more commonly than by chance in close relatives, in subjects or families with other organ-specific autoimmune diseases of the thyroid, adrenal or parathyroid, in those with premature greying, blue eyes and vitiligo, and in persons of blood group A. An association with human leucocyte antigen (HLA)-3 has been reported in some but not all series and in

Table 13.4 Causes of cobalamin deficiency causing megaloblastic anaemia.

Nutritional
Vegans
Malabsorption
Pernicious anaemia
Gastric causes
Congenital intrinsic factor deficiency or functional abnormality. Total or partial gastrectomy
Intestinal causes
Intestinal stagnant loop syndrome: jejunal diverticulosis, ileocolic fistula, anatomical blind loop, intestinal stricture, etc.
Ileal resection and Crohn's disease
Selective malabsorption with proteinuria
Tropical sprue
Transcobalamin deficiency
Fish tapeworm

Table 13.5 Malabsorption of cobalamin may occur in the following conditions but is not usually sufficiently severe and prolonged to cause megaloblastic anaemia.

Gastric causes
Simple atrophic gastritis (food cobalamin malabsorption)
Zollinger–Ellison syndrome
Gastric by-pass (bariatric) surgery
Use of proton pump inhibitors
Intestinal causes
Gluten-induced enteropathy
Severe pancreatitis
HIV infection
Radiotherapy
Graft-versus-host disease
Deficiency states
Cobalamin, folate, protein, riboflavin?, nicotinic acid?
Drug therapy
Colchicine, *p*-aminosalicylate, neomycin, slow-release potassium chloride, anticonvulsant drugs, ?metformin, ?phenformin, cytotoxic drugs
Alcohol

those with endocrine disease, with HLA-B8, -B12 and -BW15. The life expectancy has been estimated as normal in women once regular treatment has begun. Men have been reported to have a slightly lower life expectancy as a result of a higher incidence of carcinoma of the stomach than in control subjects.

Diagnosis

This is usually suspected from the clinical picture and the findings of megaloblastic anaemia due to cobalamin deficiency. Tests for circulating gastric autoantibodies and serum gastrin levels are important. The *serum gastrin* level is usually raised in PA (>200 µg/L), the hormone coming from endocrine cells in the gastric fundus. Raised serum gastrin also occurs in simple atrophic gastritis. Serum pepsinogen I levels are low (<30 µg/L) in over 90% of those affected and a low ratio of serum pepsinogen I to pepsinogen II correlates with the presence of chronic atrophic gastritis.

Gastric biopsy

This shows atrophy of all layers of the body and also fundal atrophy, with loss of glandular elements, an absence of parietal and chief cells and replacement by mucous cells, a mixed inflammatory cell infiltrate and sometimes intestinal metaplasia. The infiltrate of plasma cells and lymphocytes contains an excess of CD4 cells. The antral mucosa is usually well preserved. *Helicobacter (H.) pylori* infection is infrequent in PA, but it has been suggested that *H. pylori* gastritis may represent an early phase of atrophic gastritis, often associated with iron deficiency, which is gradually replaced, in some individuals, by an immune process with disappearance of *H. pylori* infection. Corticosteroid therapy may restore acid and IF secretion temporarily.

Antibodies to gastric antigens

IF antibodies: Two types of IF antibodies may be found in the sera of patients with PA, both being IgG. One, the 'blocking' or 'type I' antibody, prevents the combination of IF and cobalamin, whereas the other, the 'binding', 'type 2' antibody, which attaches to IF whether joined to cobalamin or not, prevents attachment of IF to ileal mucosa. Type 1 antibody occurs in the serum of about 55% of patients, type 2 antibody in 35%. IF antibodies cross the placenta and cause temporary IF deficiency in the newborn infant. Patients with PA also show cell-mediated immunity to IF. IF antibodies are rarely found in conditions other than PA. Type I antibody has been detected rarely in the sera of patients without PA but with thyrotoxicosis, myxoedema, Hashimoto disease or diabetes mellitus, and in relatives of PA patients. IF antibodies have also been detected in gastric juice in about 80% of patients with PA. These antibodies may reduce absorption of dietary cobalamin by combining with small amounts of remaining IF in the gastric juice. Achlorhydria favours the formation of this antigen–antibody complex.

Parietal cell and gastrin receptor antibodies: Parietal cell antibody is present in the sera of almost 90% of adult patients with PA, but it is frequently present in other subjects. Thus, it occurs in as many as 16% of randomly selected female subjects aged over 60 years and in a smaller proportion of younger control subjects; it is found more frequently than in control subjects in relatives of PA patients. These antibodies are also found more frequently in patients with simple atrophic gastritis, chronic active hepatitis and thyroid disorders and their relatives, as well as in Addison disease, rheumatoid arthritis and other conditions. The parietal cell antibody is directed against the α and β-subunits of the gastric proton pump (H^+/K^+ -ATPase). The sera of PA patients may also contain an autoantibody to the gastrin receptor, although this test is not used clinically.

Hypogammaglobulinaemia

PA is found more often than by chance in patients with a deficiency of IgA or with complete hypogammaglobulinaemia. These subjects resemble others with PA, except that they often present relatively early (before the age of 40 years), they have a lower incidence of serum IF and parietal cell antibodies, and they may show intestinal malabsorption. They may also have a history of recurrent infections. The gastric lesion is similar to that in other cases, except that plasma cells are absent from

the inflammatory cell infiltrate and the antrum is involved. Serum gastrin levels are normal.

Juvenile pernicious anaemia

This usually occurs in older children and resembles PA in adults. Gastric atrophy, achlorhydria and serum IF antibodies are all present, although parietal cell antibodies are usually absent. About half of these patients show an associated endocrinopathy such as autoimmune thyroiditis, Addison disease or hypoparathyroidism; in some, mucocutaneous candidiasis occurs.

Congenital intrinsic factor deficiency or functional abnormality

The affected child usually shows no demonstrable IF but has a normal gastric mucosa and normal secretion of acid. The inheritance is autosomal recessive. These patients usually present with megaloblastic anaemia in the first, second or third year of life when stores of cobalamin accumulated from the mother *in utero* are used up; a few have presented as late as the second decade. Parietal cell and IF antibodies are absent. Variants have been described in which the child is born with IF that can be detected immunologically, but which is unstable or functionally inactive, being unable either to bind cobalamin or to facilitate its uptake by the ileum.

Gastrectomy

Following total gastrectomy, cobalamin deficiency is inevitable and prophylactic cobalamin therapy should be commenced immediately following the operation. After partial gastrectomy, 10–15% of patients also develop this deficiency. The exact incidence and time of onset are most influenced by the size of the resection and the pre-existing size of the cobalamin body store. Gastric plication for obesity (bariatric surgery) may also cause malabsorption of cobalamin.

Simple atrophic gastritis (food cobalamin malabsorption)

The normal IF-mediated mechanism of cobalamin absorption requires adequate gastric output of acid and pepsin to ensure the release of food cobalamin. Failure of this mechanism is believed to be responsible for a condition more common in the elderly known as *food cobalamin malabsorption*. The syndrome has also been described in association with *H. pylori* infection, long-term use of histamine H_2 -receptor antagonists and proton pump inhibitors, chronic alcohol excess, pancreatic exocrine failure, Sjögren syndrome and systemic sclerosis. The syndrome is associated with low serum cobalamin levels, with or without evidence of cobalamin deficiency, such as raised serum levels of methylmalonic acid and homocysteine. A minority of patients with food cobalamin malabsorption may go on to develop clinically significant cobalamin deficiency, but the frequency of occurrence and reasons for this progression are not clear.

Intestinal causes of cobalamin malabsorption

Malabsorption of cobalamin occurs in a variety of intestinal lesions in which there is colonisation of the upper small intestine by faecal organisms. This may occur in patients with jejunal diverticulosis, entero-anastomosis, intestinal stricture or fistula, or with an anatomical blind loop due to Crohn's disease, tuberculosis or an operative procedure. Bacterial overgrowth in the small intestine may also cause spurious elevation of serum methylmalonate (see further on). Removal of 1.2 m or more of terminal ileum causes malabsorption of cobalamin. In some patients, following ileal resection, particularly if the ileocaecal valve is incompetent, colonic bacteria may contribute further to cobalamin deficiency.

Nearly all patients with acute and subacute tropical sprue show malabsorption of cobalamin; this may persist as the principal abnormality in the chronic form of the disease, when the patient may present with megaloblastic anaemia or neuropathy due to cobalamin deficiency. Absorption of cobalamin usually improves after antibiotic therapy and, in the early stages, after folic acid therapy. Malabsorption of cobalamin occurs in about 30% of untreated patients with gluten-induced enteropathy and correlates with the degree of steatorrhoea. Cobalamin deficiency is not usually severe in these patients and is probably never the cause of megaloblastic anaemia unless another lesion causing malabsorption of cobalamin (e.g. stagnant loop syndrome) is present. The absorption improves when these patients are treated with a gluten-free diet.

Selective malabsorption of cobalamin with proteinuria (also known as Imerslünd syndrome, Imerslünd–Grasbeck syndrome, megaloblastic anaemia Type 1 (MGA1) is an autosomal recessive disease and is the most common cause of megaloblastic anaemia due to cobalamin deficiency in infancy in Western countries. There are familial clusters in Finland, Norway, the Middle East and North Africa. The patients usually present with anaemia between the ages of 1 and 5 years, and secrete normal amounts of IF and gastric acid. In some cases, such as in Finland, impaired synthesis, processing or ligand binding of cubilin due to inherited mutations, for example c.391C→T (named FM1) and a mutation at an intron causing a truncated protein (FM2), have been implicated. In others, for example in Norway, mutation of the gene for AMN has been reported. Other tests of intestinal absorption are normal. Over 90% of these patients show non-specific proteinuria but renal function is otherwise normal and renal biopsy has not shown any consistent renal defect. A few of these patients have shown aminoaciduria and congenital renal abnormalities, such as duplication of the renal pelvis.

The fish tapeworm (*Diphyllobothrium latum*) lives in the small intestine of humans and accumulates cobalamin from food, rendering this unavailable for absorption. People acquire the worm by eating raw or partly cooked fish. Infestation is common around the lakes of Scandinavia, Germany, Japan, North America and Russia. Megaloblastic anaemia or cobalamin neuropathy occurs only in those with a heavy infestation, with the worm high in the small intestine. Many carriers have no cobalamin deficiency.

In severe chronic pancreatitis, lack of trypsin is thought to be why dietary cobalamin attached to gastric non-IF (R) binder is unavailable for absorption. It has also been proposed that in pancreatitis, the concentration of calcium ions in the ileum falls below the level needed to maintain normal cobalamin absorption. Serum cobalamin levels tend to fall in patients with HIV infection and are subnormal in 10–35% of those with AIDS. Malabsorption of cobalamin not corrected by IF has been shown in some, but not all, patients with subnormal serum cobalamin levels. Malabsorption of cobalamin has been reported in Zollinger–Ellison syndrome. It is thought that there is a failure to release cobalamin from R binding protein due to inactivation of pancreatic trypsin by high acidity, as well as interference with IF binding of cobalamin.

Both total body irradiation and local radiotherapy to the ileum (e.g. as a complication of radiotherapy for carcinoma of the cervix) may cause malabsorption of cobalamin. Graft-versus-host disease commonly affects the small intestine: malabsorption of cobalamin due to abnormal gut flora, as well as damage to ileal mucosa, is frequent. Some drugs cause malabsorption of cobalamin, but rarely megaloblastic anaemia (Table 13.5). Metformin may lower serum cobalamin by lowering TCI. The use of histamine H_2-blockers for treatment of peptic ulcer disease causes decreases in cobalamin absorption, and continued use may lower serum cobalamin level. Other causes of cobalamin malabsorption are listed in Table 13.5.

Abnormalities of cobalamin metabolism: congenital transcobalamin deficiency or abnormality

Infants with TC deficiency usually present with megaloblastic anaemia within a few weeks of birth. Serum cobalamin and folate levels are normal, but the anaemia responds to massive (e.g. 1 mg 3 times weekly) injections of cobalamin, which cause free cobalamin to enter marrow cells by passive diffusion in the absence of functional TC. Some cases show neurological complications. In some cases, the protein is present in normal amounts but is unable to bind cobalamin or to attach to the cell surface and so is functionally inert. Genetic abnormalities found include mutations of an intra-exonic cryptic splice site, extensive or single nucleotide deletion, nonsense mutation and an RNA editing defect. These infants do not show methylmalonic aciduria, but malabsorption of cobalamin occurs in all cases and reduced immunoglobulins in some. Less severe cases present later in childhood. Failure to institute adequate cobalamin therapy or treatment with folic acid may lead to neurological damage.

Congenital methylmalonic acidaemia and aciduria

Infants with this abnormality are ill from birth, with vomiting, failure to thrive, severe metabolic acidosis, ketosis and intellectual disability. Anaemia, if present, is normocytic and normoblastic. The condition may arise as a result of a functional defect in either the mitochondrial methylmalonyl-CoA mutase or its co-factor adocobalamin (Figure 13.2). Mutations in methylmalonyl-CoA mutase are not responsive, or only poorly responsive, to treatment with cobalamin. Two disorders result in cobalamin-responsive methylmalonic acidaemia. In cobalamin (Cbl)A disease, there is failure of reduction of cobalamin III (Cbl^{3+}) or cobalamin II (Cbl^{2+}) to cobalamin I (Cbl^{1+}) in mitochondria; in CblB disease, there is a defect of an adenosyltransferase required for synthesis of ado-cobalamin (Figure 13.2). A proportion of infants with CblA and CblB disease respond to cobalamin in large doses, whereas others are unresponsive. In those who do not respond to cobalamin, the enzyme methylmalonyl-CoA mutase is lacking (mut^0) or defective (mut^-). Some children have combined methylmalonic aciduria and homocystinuria due to defective formation of both cobalamin co-enzymes. The defects are in the transfer of cobalamin from the endocytic compartment of lysosomes to the cytoplasm (CblF disease) or in the reduction of cobalamin 3^+ to cobalamin 2^+ after transfer to the cytoplasm (CblC and CblD diseases). Over 100 cases of CblC disease have been described. It usually presents in the first year of life with feeding difficulties, developmental delay, microcephaly, seizures, hypotonia and megaloblastic anaemia.

Some patients present with homocystinuria and megaloblastic anaemia, often with neurological defects, but without methylmalonic aciduria. There is a selective deficiency of methylcobalamin. These conditions have been termed CblE and CblG disease and are due to lack of association of methylcobalamin with methionine synthase.

Acquired abnormality of cobalamin metabolism: nitrous oxide inhalation

N_2O irreversibly oxidises methylcobalamin from its active, fully reduced Cbl^{1+} state to an inactive Cbl^{2+} precursor. This has been shown to inactivate methylcobalamin and methionine synthase. This occurs in both humans and experimental animals and was of importance in the megaloblastic anaemia that occurred in patients undergoing prolonged N_2O anaesthesia (e.g. in intensive care units). A neuropathy resembling cobalamin neuropathy has

been described in dentists and anaesthetists who are repeatedly exposed to N_2O and in monkeys exposed to the gas for many months. Recovery from N_2O exposure requires regeneration of methionine synthase, as this protein is damaged by active oxygen derived from the N_2O–cobalamin reaction.

Diagnosis of cobalamin deficiency

The diagnosis of cobalamin or folate deficiency has traditionally depended on the recognition of the relevant abnormalities in the peripheral blood and/or bone marrow and subsequent analysis of the blood levels of the vitamins. Other causes of macrocytosis and hypersegmented neutrophils are listed in Table 13.3. However, assays of serum methylmalonic acid and homocysteine (see further on) have shown these to be raised in some subjects without haematological abnormalities, including a proportion with normal levels of serum cobalamin and folate in whom, nevertheless, the levels of the metabolites fall to normal with cobalamin and/or folate therapy. The significance of these biochemical changes remains controversial. They may imply functional cobalamin or folate deficiency, also termed insufficiency, not reflected by subnormal levels of the vitamins or by disturbed haemopoiesis. If so, it would imply that the accepted normal serum and red cell levels of the vitamins reflect body stores that are sufficiently high to prevent haematological changes, but which in some subjects may not be optimal for prevention of other complications of the deficiencies, including neurological decline and NTDs in the fetus. The extensive review of Smith, Warren and Refsum (2018) addresses these issues in relation to cobalamin.

Measurement of serum cobalamin

Serum cobalamin is usually measured by one of a number of enzyme-linked immunosorbent assays or by a two-step chemiluminescent microparticle immunoassay (CMIA). In this assay serum B12 competes with a B12-acridium-labelled conjugate for binding to intrinsic factor-coated paramagnetic microparticles, the higher the serum B12 the lower the final luminescence of the intrinsic factor-conjugate complex. These assays are frequently automated. Normal serum cobalamin levels range in different assays from 160–200 ng/L to about 1000 ng/L (ng × 0.738 = pmol; 200 ng/L = 148 pmol/L). In patients with megaloblastic anaemia due to cobalamin deficiency, the level was usually less than 100 ng/L (74 pmol/L) with the earlier microbiological assays but is subnormal but more variable with the more modern immunosorbent assays. In general, the more severe the deficiency, the lower the serum cobalamin level. In patients with spinal cord damage due to the deficiency, levels are usually very low even in the absence of anaemia.

Subnormal or low normal serum cobalamin levels without haematological or vitamin B12 neuropathy are found most frequently in vegans, the elderly and in patients with other causes of mild cobalamin deficiency (Table 13.5). The significance of these findings and whether treatment should be given are discussed above and in the treatment section of this chapter.

Subnormal levels may also occur in pregnancy, returning to normal after delivery, and in patients with megaloblastic anaemia due to folate deficiency, which correct along with the anaemia with folic acid therapy. They are also found without haematological or other clinical manifestations in patients with mutations of the *TCN1* gene that codes for HC (TCI). False normal levels have been described with some of the assays in patients with IF antibodies in serum. The relative concentrations of HC and TC also influence the total serum cobalamin level. False normal levels have been described in patients with megaloblastic anaemia due to cobalamin deficiency if serum HC is raised, as in the myeloproliferative neoplasms. Raised serum cobalamin levels (if not due to recent therapy) are usually due to a rise in HC in these haematological neoplastic diseases or to liver or renal disease with increased saturation of HC and TC.

Serum holotranscobalamin (holoTCII, holoTC)

Since TC is the plasma cobalamin transport protein that is responsible for cellular uptake and delivery of cobalamin, the idea was put forward that measurement of only circulating cobalamin that was bound to TC (holoTC) would provide a more meaningful measure of cobalamin status than total serum cobalamin. Commercial kits for measurement by immunoassay of holoTC (also called 'active B12' or 'active cobalamin') are now available and used diagnostically. The expected values in healthy individuals are from 35–171 pmol/L. The lower limit of the normal range given by the manufacturers of commercial kits 19–42 pmol/L and the upper limit form 134–157 pmol/L. The assays are considered to have a narrower 'grey zone' (uncertainty range) than serum cobalamin assays, to be more sensitive and specific.

Serum methylmalonate and homocysteine levels

In patients with cobalamin deficiency, the serum methylmalonate (MMA) and homocysteine levels are raised. Sensitive methods for measuring MMA and homocysteine in serum have been introduced and recommended for the early diagnosis of cobalamin deficiency, even in the absence of haematological abnormalities or subnormal levels of serum cobalamin or folate. However, serum MMA fluctuates in patients with renal failure. Mildly elevated serum MMA and/or homocysteine levels occur in up to 30% of apparently healthy volunteers, with serum cobalamin levels up to 350 ng/L and normal serum folate levels and in 15% of elderly subjects, even with cobalamin levels above 350 ng/L. These findings bring into question the exact cut-off points for normal MMA and homocysteine levels. It is also unclear at present whether these mildly raised metabolite levels have clinical consequences and how many of the subjects will

progress to clinically overt cobalamin deficiency. When cobalamin supplies to the cell are suboptimal, there may be preferential use of methylcobalamin for methionine synthesis compared with adocobalamin for MMA metabolism. Urinary MMA excretion may also be used to screen for cobalamin deficiency, but this is also increased in aminoaciduria (e.g. Fanconi syndrome).

Homocysteine exists in plasma as single molecules, as two molecules linked together (homocystine) and as mixed homocysteine–cysteine disulfides. Serum homocysteine levels are raised in both early cobalamin and folate deficiency, but they may be raised in other conditions, for example chronic renal disease, alcohol excess, smoking, pyridoxine deficiency, hypothyroidism, therapy with steroids, ciclosporin and other drugs. Levels are also higher in serum than in plasma, in men than in premenopausal women, in women taking hormone replacement therapy or oral contraceptive users and in elderly subjects and patients with several inborn errors of metabolism affecting enzymes in trans-sulfuration pathways of homocysteine metabolism. Thus, homocysteine levels are not widely used for diagnosis of cobalamin or folate deficiency.

Tests for the cause of cobalamin deficiency

The diet history reveals whether the subject is vegan or a strict vegetarian. Studies of cobalamin absorption were used, but because of the unavailability of radioactive cobalamin have become obsolete. Serum tests for gastrin and antibodies to parietal cells and intrinsic factor aid in the diagnosis of PA. Upper GI endoscopy, including gastric biopsy, helps to confirm the diagnosis and exclude gastric neoplasms. A history of gastric or ileal surgery and small intestinal imaging studies may point to a small intestinal cause of cobalamin malabsorption.

Folate

Dietary folate

Folic acid (pteroylglutamic acid) is a yellow, crystalline and water-soluble substance (molecular weight 441 kDa). It is the parent compound of a large family of folate compounds. Pteroylglutamic acid consists of three parts: pteridine, *p*-aminobenzoate and l-glutamic acid (Figure 13.7). It is only a minor component of normal food folates (probably less than 1%), which differ from it in three respects (Figure 13.7): (i) they are partly or completely reduced at positions 4, 5, 7 and 8 in the pteridine portion to dihydrofolate or THF derivatives; (ii) they usually contain a single carbon unit of varying degrees of reduction, such as a methyl group at N-5 or N-10; and (iii) 70–90% of natural folates contain a chain of three or more glutamate residues linked to each other by the unusual γ-peptide bond and are called pteroyl- or folate-polyglutamates (Figure 13.7). In human cells, four, five and six glutamate residues are usual.

Most foods contain some folate. The highest concentrations are found in liver and yeast (>200 μg per 100 g), spinach, other greens and nuts (>100 μg per 100 g). The total folate content of an average Western diet is about 250 μg daily, but the amount varies widely according to the type of food eaten and the method of cooking. Folate is easily destroyed by heating, particularly in large volumes of water; over 90% may be lost.

Body stores and requirements

Total body folate in the adult is about 10 mg, the liver containing the largest store. Daily adult requirements are about 100–200 μg. Up to 13 μg of folate is lost as such in the urine each day, but breakdown products of folate are also lost in urine. Losses of

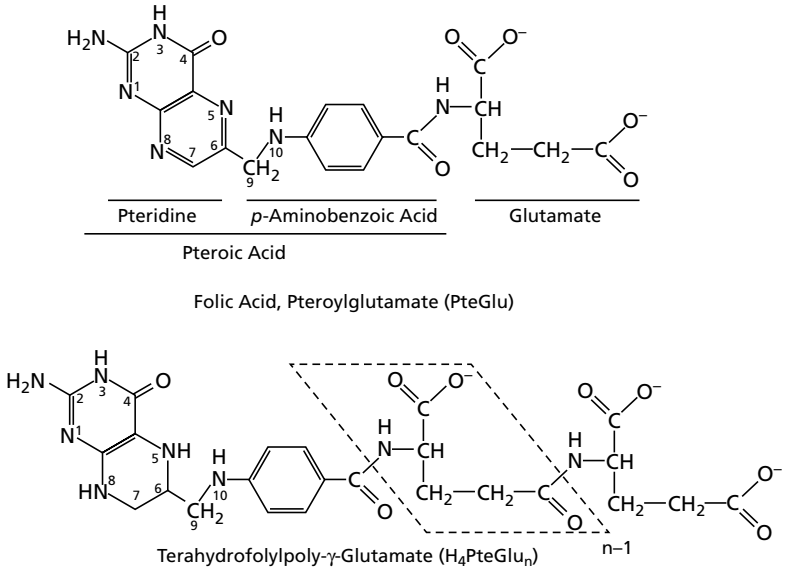


Figure 13.7 The structure of folic acid (pteroylglutamic acid) and of a tetrahydrofolate polyglutamate.

folate also occur in sweat and skin; faecal folate is largely derived from colonic bacteria. Stores are only sufficient for about 4 months in normal adults, so severe folate deficiency may develop rapidly.

Absorption

The principal site of folate absorption is the upper small intestine, and there is a steep fall-off in absorptive capacity in the lower jejunum and ileum. The absorption of all forms tested is rapid, with a rise in blood level occurring within 15–20 min of ingestion.

The small intestine has a tremendous capacity to absorb folate monoglutamates: about 90% of a single dose is absorbed, regardless of whether this is a small (100 μg) or large (15 mg) amount. A proton-coupled high-affinity folate transporter with a low pH optimum, termed PCFT/HCP1, is located at the apical brush border of the duodenal, and to a lesser extent jejunal mucosa and in other cells, including the blood–brain barrier. It accounts for the bulk of folate absorption, including folic acid itself, and loss of function in hereditary folate malabsorption is not compensated by other folate transporters expressed on intestinal cells.

The absorption of folate polyglutamates with higher numbers of glutamate residues is less. This may be due to the limited capacity of the small intestine to hydrolyse these compounds or to their limited transfer in the mucosal cell. On average, about 50% of food folates is absorbed.

Polyglutamate forms are hydrolysed to monoglutamate derivatives, either in the lumen of the intestine or within the mucosa; they do not enter portal blood intact. Monoglutamate or polyglutamate forms of dietary folate, which are already partly or completely reduced, are converted to 5-methyl-THF within the small intestinal mucosa before entering the portal plasma. The monoglutamates are actively transported across the enterocyte by a carrier-mediated mechanism. Pteroylglutamic acid at doses greater than 400 μg is absorbed largely unchanged and converted to natural folates in the liver. Doses <400 μg are largely converted to 5-methyl-THF during absorption through the intestine.

Enterohepatic circulation

About 60–90 μg of folate enters the bile each day and is excreted into the small intestine. Loss of this folate, together with the folate of sloughed intestinal cells, accelerates the speed with which folate deficiency develops in malabsorption conditions.

Transport

Folate is transported in plasma, about one-third loosely bound to albumin and two-thirds unbound. In all body fluids (plasma, cerebrospinal fluid, milk, bile) folate is largely, if not entirely, 5-methyl-THF in the monoglutamate form. A carrier-mediated active process is involved in the entry of folate into cells, the rate of uptake being linked to the rate of folate polyglutamate synthesis in the cell, which in replicating cells is related to the rate of DNA synthesis. In most cells, folates are retained with tight binding to folate-binding proteins, three of which are enzymes involved in methyl group metabolism (sarcosine dehydrogenase, dimethylglycine dehydrogenase and glycine *N*-methyltransferase), until the cell dies. Intact liver cells can release folate. Two types of folate-binding protein are involved in entry of methyl-THF into cells. The reduced folate carrier SLC19A1 is a facilitative transporter with a pH optimum of 7.4 and the characteristics of an anion exchanger. Two glycosylphosphatidylinositol (GPI)-linked folate receptors mediate cellular folate uptake by an endocytic mechanism, with internalisation in a vesicle (caveola), which is then acidified, releasing folate into the vesicle lumen. Folate is then carried by the membrane folate transporter PCFT/HCPI into the cytoplasm; the caveola recycles to the cell surface, where its high-affinity receptors are reutilised. The GPI-linked transporters may be involved in transport of oxidised folates and folate breakdown products to the liver for excretion in bile.

Biochemical functions

Folates (as the intracellular polyglutamate derivatives) act as co-enzymes in the transfer of single-carbon units from one compound to another (see Figure 13.3 and Table 13.2). Two of these reactions are involved in purine and one in pyrimidine synthesis necessary for DNA and RNA replication. Folate is co-enzyme for methionine synthesis, in which cobalamin is also involved and THF is generated. THF, the substrate for polyglutamate synthesis, is also the acceptor of single-carbon units newly entering the active pool via conversion of serine to glycine. Methionine, the other product of the methionine synthase reaction, is the precursor for SAM, the universal methyl donor involved in over 100 methyltransferase reactions.

During thymidylate synthesis, 5,10-methylene-THF is converted to dihydrofolate (Figure 13.3). The enzyme dihydrofolate reductase converts this to THF. The drugs methotrexate, pyrimethamine and, mainly in bacteria, trimethoprim inhibit dihydrofolate reductase, and this prevents formation of the active folate co-enzymes from dihydrofolate. A small fraction of the folate co-enzyme is not recycled during thymidylate synthesis but is degraded at the C-9–N-10 bond.

Causes of folate deficiency

Nutritional

Dietary folate deficiency is common (Table 13.6). Indeed, in most patients with folate deficiency, a nutritional element is present. Certain individuals are particularly at risk of having diets

Table 13.6 Causes of folate deficiency.

Dietary
Particularly in old age, infancy, poverty, alcohol use disorder, and some forms of psychiatric illness; may be associated with scurvy or kwashiorkor
Malabsorption
Major causes of deficiency
Tropical sprue, gluten-induced enteropathy in children and adults, and in association with dermatitis herpetiformis, specific malabsorption of folate, intestinal megaloblastosis caused by severe cobalamin or folate deficiency
Minor causes of deficiency
Extensive jejunal resection, Crohn's disease, partial gastrectomy, congestive heart failure, Whipple disease, scleroderma, amyloid, diabetic enteropathy, systemic bacterial infection, lymphoma, sulfasalazine
Excess utilisation or loss
Physiological
Pregnancy and lactation, prematurity
Pathological
Haematological diseases: chronic haemolytic anaemias, sickle cell anaemia, thalassaemia major, myelofibrosis
Malignant diseases: carcinoma, lymphoma, leukaemia, myeloma
Inflammatory diseases: tuberculosis, Crohn's disease, psoriasis, exfoliative dermatitis, malaria
Metabolic disease: homocystinuria
Excess urinary loss: congestive heart failure, active liver disease
Haemodialysis, peritoneal dialysis
Antifolate drugs
Anticonvulsant drugs (phenytoin, primidone, barbiturates), sulfasalazine
Nitrofurantoin, tetracycline, anti-tuberculosis (less well documented)
Mixed causes
Liver diseases, alcohol use disorder, intensive care units

In severely folate-deficient patients with causes other than those listed under Dietary, poor dietary intake is often present.

containing inadequate amounts of folate, including the elderly, edentulous, poor, those with alcohol use disorder and some psychiatric illnesses, and those who have undergone gastric surgery. With total cessation of intake or absorption, depletion of stores will occur in 3–6 months. In the USA and other countries, where fortification of the diet with folic acid has been adopted to reduce the incidence of NTDs, the prevalence of folate deficiency has dropped dramatically and is now almost restricted to high-risk groups with increased folate needs. Nutritional folate deficiency occurs in kwashiorkor and scurvy, and in infants with repeated infections or who are fed solely on goats' milk, which has a low folate content (6 μg/L) compared with human or cows' milk (50 μg/L).

Malabsorption

Malabsorption of dietary folate occurs in tropical sprue and in gluten-induced enteropathy in children and in adults. In the rare recessive congenital syndrome of selective malabsorption of folate, there is an associated defect of folate transport into the cerebrospinal fluid, and these patients show megaloblastic anaemia from the age of a few months, responding to physiological doses of folic acid given parenterally but not orally, or large oral doses of 5-formyl-THF. They show intellectual disability, convulsions and other central nervous system abnormalities. Loss-of-function mutations, usually homozygous in the gene coding for the low pH transporter PCFT/HCPI, underlie the disease. Minor degrees of malabsorption may also occur following jejunal resection or partial gastrectomy, in Crohn's disease and in systemic infections but, in these conditions, if severe deficiency occurs, it is usually largely due to poor nutrition.

Malabsorption of folate has been described in patients receiving sulfasalazine, cholestyramine and triamterene. It has also been associated with anticonvulsant drug therapy and alcohol excess. In the intestinal stagnant loop syndrome, the predominant effect of the small intestinal bacteria is to cause a rise in serum, red cell and urinary folate by synthesising folate, which is then absorbed.

Excess utilisation or loss

Pregnancy

Folate requirements are increased by 200–300 μg to about 400 μg daily in a normal pregnancy, partly because of transfer of the vitamin to the fetus, but mainly because of increased folate catabolism due to cleavage of folate co-enzymes in rapidly proliferating tissues. Megaloblastic anaemia due to this deficiency is now largely prevented by prophylactic folic acid therapy. It occurred in 0.5% of pregnancies in the UK and other Western countries, but the incidence is much higher in countries where the general nutritional status is poor. The deficiency is more common in pregnant women who, because of an inadequate diet, are also iron deficient than in those with normal iron stores. Occasionally, when there is an associated infection, acute arrest of haemopoiesis with pancytopenia may occur; this resembles aplastic anaemia, except that the marrow shows obvious megaloblastic changes.

A number of consequences of folate deficiency in pregnancy have been described, including antenatal and postpartum

haemorrhages, prematurity and congenital malabsorption in the fetus. These have not been fully established, but many studies have shown that prophylactic folic acid therapy reduces the incidence of NTDs.

Prematurity

The newborn infant has higher serum and red cell folate concentrations than the adult, but the newborn infant's demand for folate has been estimated to be up to 10 times that of adults on a weight basis and the neonatal folate level falls rapidly to the lowest values at about 6 weeks of age. The falls are steepest and liable to reach subnormal levels in premature babies, a number of whom develop megaloblastic anaemia responsive to folic acid at about 4–6 weeks of age. This occurs particularly in the smallest babies (<1500 g birth weight) and in those who have feeding difficulties or infections, or who have undergone multiple exchange transfusions. In these babies, prophylactic folic acid should be given.

Haematological disorders

Folate deficiency frequently occurs in chronic haemolytic anaemia and other conditions of increased cell turnover because it is not completely reutilised after performing co-enzyme functions, and it is partly lost as pteridines. Patients with primary myelofibrosis may develop folate deficiency at some stage of the illness. There is also a high incidence of mild folate deficiency in patients with forms of leukaemia, lymphoma, myeloma or carcinoma, although it is unusual for this to progress to megaloblastic anaemia. Treatment with folic acid should be avoided (as it may 'feed' the tumour) unless severe megaloblastic anaemia due to folate deficiency is clinically important.

Inflammatory conditions

Chronic inflammatory diseases, such as tuberculosis, rheumatoid arthritis, Crohn's disease, psoriasis, exfoliative dermatitis, bacterial endocarditis and chronic bacterial infections, cause deficiency by reducing the appetite and by increasing the demand for folate. Systemic infections may also cause malabsorption of folate. Severe deficiency is virtually confined to those patients with the most active disease and the poorest diet. Fever *per se* has also been suggested to interfere with folate metabolism by inhibiting temperature-dependent folate enzymes. In patients with subclinical folate deficiency from causes other than infections, intercurrent infections may precipitate severe megaloblastic anaemia.

Homocystinuria

This is a rare metabolic defect in the conversion of homocysteine to cystathionine. Folate deficiency occurring in most of these patients may be due to excessive utilisation because of compensatory increased conversion of homocysteine to methionine.

Long-term dialysis

As folate is only loosely bound to plasma proteins, it is easily removed from plasma by haemodialysis or peritoneal dialysis (in contrast, cobalamin is not removed from plasma by dialysis as it is firmly protein bound). The amount of body folate that can be removed in this way is relatively small. Nevertheless, in patients with anorexia, vomiting, infections and haemolysis, folate stores may become depleted and megaloblastic anaemia can supervene. Routine folate prophylaxis is now given.

Congestive heart failure, liver disease

Excess urinary folate losses of more than 100 µg per day may occur in some of these patients. The explanation appears to be release of folate from damaged liver cells.

Antifolate drugs

A large number of people with epilepsy who are receiving long-term therapy with phenytoin (Dilantin) or primidone (Mysoline), develop low serum and red cell folate levels. In some of these patients, megaloblastic anaemia supervenes. A number of mechanisms have been suggested: inhibition of folate absorption, inhibition of the action or synthesis of folate-dependent enzymes, displacement of folate from its plasma transport protein and induction of folate-utilising enzymes. A dietary element is present in the patients with the severest deficiencies.

Alcohol may also be a folate antagonist, as patients who are drinking spirits may develop megaloblastic anaemia that will respond to normal quantities of dietary folate or physiological doses of folic acid only if the alcohol is withdrawn. Chronic alcohol intake is associated with macrocytosis even when folate levels are normal. Inadequate folate intake is the major factor in the development of deficiency in individuals with alcohol use disorder who drink spirits. Beer is relatively folate-rich in some countries, depending on the technique used for brewing.

The drugs that inhibit dihydrofolate reductase include methotrexate, pyrimethamine and trimethoprim. Methotrexate has the most powerful action against the human enzyme, whereas trimethoprim is most active against the bacterial enzyme and is only likely to cause megaloblastic anaemia when used in conjunction with sulfamethoxazole in patients with pre-existing folate or cobalamin deficiency. The activity of pyrimethamine is intermediate. The antidote to these drugs is folinic acid (5-formyl-THF).

Congenital abnormalities of folate metabolism

A number of infants have been described with congenital defects of folate enzymes (e.g. cyclohydrolase or methionine synthase). Some had megaloblastic anaemia.

Diagnosis of folate deficiency

Serum folate

This is measured by an enzyme-linked immunosorbent assay (ELISA) or as for vitamin B12 a two-step chemiluminescent immunoassay (CMIA). In this serum folate is bound to folate binding protein coated paramagnetic microparticles. Pteroic acid acridium-labelled conjugate is then added. The final strength of chemiluminescence from the conjugate is in inverse relationship with the serum folate. Serum folate is low in all folate-deficient patients. In most laboratories, the normal range is from 3.0 μg/L (16.5 nmol/L) to about 15 μg/L (82.5 nmol/L). The serum folate is markedly affected by recent diet; inadequate intake for as little as 1 week may cause the level to become subnormal.

The serum folate level rises in severe cobalamin deficiency because of blockage in conversion of methyl-THF, the major circulating form, to THF; raised levels have also been reported in the intestinal stagnant loop syndrome, acute kidney injury and active liver damage. (High levels are also obtained when the patient is taking folic acid dietary supplementation or therapy, or, if the blood sample is haemolysed, because of the high concentration of folate in red cells.)

Red cell folate

The red cell folate assay is a valuable test of body folate stores. It is less affected by recent diet and traces of haemolysis than is the serum assay. In normal adults, concentrations range from 160 to 640 μg/L of packed red cells. Subnormal levels not only occur in patients with megaloblastic anaemia due to folate deficiency, but also occur in nearly two-thirds of patients with megaloblastic anaemia due to cobalamin deficiency. If cobalamin deficiency is excluded, however, a low red cell folate can be used as an indication that severe folate deficiency is present and warrants full investigation and treatment. False normal results may occur if the folate-deficient patient has received a recent blood transfusion or if the patient has a raised reticulocyte count (e.g. due to haemorrhage or haemolytic anaemia).

Serum homocysteine

See previously

General management of megaloblastic anaemia

It is usually possible to establish which of the two deficiencies, folate or cobalamin, is the cause of the anaemia and to treat only with the appropriate vitamin. In patients who enter hospital severely ill, however, it may be necessary to treat with both vitamins in large doses once blood samples have been taken for cobalamin and folate assay and a bone marrow has been performed (if deemed necessary). Transfusion is usually unnecessary and inadvisable. If it is essential, packed red cells should be given slowly and one or two units will be ample. Exchange transfusion, as well as the usual treatment for heart failure, should be considered in patients with extreme anaemia and congestive heart failure. Platelet concentrates are of value in reducing spontaneous bleeding in rare patients with severe thrombocytopenia.

Treatment of cobalamin deficiency

It is usually necessary to treat patients who have developed cobalamin deficiency with haematological and neurological manifestations with lifelong regular cobalamin therapy. In the UK, the form used is hydroxocobalamin; in the USA, cyanocobalamin is used. In a few instances, the underlying cause of cobalamin deficiency can be permanently corrected, for instance, the fish tapeworm, tropical sprue or an intestinal stagnant loop that is amenable to surgery.

The indications for starting cobalamin therapy are well-documented megaloblastic anaemia or neuropathy due to the deficiency. It is also necessary to treat any patients with haematological abnormalities due to cobalamin deficiency, even in the absence of anaemia (e.g. hypersegmented neutrophils, macrocytosis or megaloblastic erythropoiesis). Patients with borderline serum cobalamin levels, but no haematological or clinical abnormality, negative tests for parietal and intrinsic factor antibodies and with normal serum gastrin levels should, if practicable, be followed, for example at yearly intervals, to ensure that the cobalamin deficiency does not progress. If serum homocysteine levels are also raised hydroxocobalamin, for example 1 mg every 6 months or oral cyanocobalamin can be started. Cobalamin should be given routinely to all patients who have had a total gastrectomy or ileal resection. Patients who have undergone gastric reduction for control of obesity or who are receiving long-term treatment with proton pump inhibitors should be screened and given cobalamin replacement as necessary.

Cobalamin therapy has been used in a wide range of conditions such as the chronic fatigue syndrome, multiple sclerosis and other neurological diseases with normal serum cobalamin levels and no haematological evidence of deficiency. There are no controlled trials showing that any benefit is more than a placebo effect. It seems appropriate, however, to treat with cobalamin, either oral or intramuscular, elderly people with cognitive decline, subnormal cobalamin or low normal cobalamin levels and raised serum homocysteine levels. Controlled clinical trials are, however, needed to confirm this approach in order to establish more clearly which patients will benefit, whether folic acid as well as cobalamin is needed.

Replenishment of body stores should be complete with six 1 mg intramuscular injections of hydroxocobalamin given at 3–7-day intervals. More frequent doses for a more prolonged period are usually used in patients with cobalamin neuropathy, but there is no evidence that these produce a better

response. For maintenance therapy, hydroxocobalamin 1 mg intramuscularly once every 3 months is satisfactory. In the USA, hydroxocobalamin has not yet been approved for routine cobalamin replacement and cyanocobalamin is used. Because of the poorer retention of cyanocobalamin, maintenance treatment protocols generally use higher and more frequent doses (1 mg I.M. monthly). Toxic reactions to cobalamin therapy are extremely rare and are usually due to contamination in its preparation rather than to cobalamin itself.

Even when there is complete failure of the physiological IF-dependent mechanism, large daily oral doses (1 mg) of cyanocobalamin can be used for replacement and maintenance of normal cobalamin status, for example in those who cannot have injections. If this approach is used, it is important to monitor compliance and serum B12 levels, for example annually, particularly with elderly forgetful patients. Sublingual and nasal routes have also been proposed, but no long-term follow-up data are available.

Treatment of folate deficiency

There is probably never any need to give folic acid parenterally, except in patients receiving parenteral nutrition who cannot swallow tablets. Oral doses of 5–15 mg folic acid daily are satisfactory, as sufficient folate is absorbed from these extremely large doses even in patients with severe malabsorption. The length of time therapy must be continued depends on the underlying disease. It is customary to continue therapy for about 4 months when all folate-deficient red cells will have been eliminated and replaced by new folate-replete populations.

Before large doses of folic acid are given, cobalamin deficiency must be excluded and, if present, corrected, otherwise cobalamin neuropathy may develop as the cobalamin deficiency progresses, despite response of the anaemia of cobalamin deficiency to folate therapy. Long-term folic acid therapy as 5 mg weekly is required when the underlying cause of the deficiency cannot be corrected and the deficiency is likely to recur, for instance in chronic haemolytic anaemias such as thalassaemia major and sickle cell anaemia, and in primary myelofibrosis. It may also be necessary for gluten-induced enteropathy if this does not respond to a gluten-free diet. Where mild but chronic folate deficiency occurs, it is preferable to encourage improvement in the diet after correcting the deficiency with a short course of folic acid. In any patient receiving long-term folic acid therapy, it is important to measure the serum cobalamin level at regular (e.g. once yearly) intervals to exclude the coincidental development of cobalamin deficiency.

Folinic acid (5-formyl-THF)

This is a stable form of fully reduced folate. It is given orally or parenterally to overcome the toxic effects of methotrexate or other dihydrofolate reductase inhibitors (as present in co-trimoxazole).

Prevention of neural tube defects

Fortification of the diet with folic acid

In over 80 countries, food is fortified with folic acid (in grain or flour) to reduce the incidence of NTDs. In Europe this has not occurred, partly due to unfounded concern that fortification would delay the diagnosis of pernicious anaemia and allow a neuropathy to develop. The levels of fortification recommended are below those which would elicit a haematological response in cobalamin deficiency; particularly since small amounts of folic acid (<400 ug) in any meal is largely converted by the gut to methyl-THF, which cannot be used by cobalamin deficient cells. Studies have shown no exacerbation by high blood folate levels of metabolic abnormalities or of cognitive impairment in subjects with low vitamin B12 status. Moreover there has been no increase in the proportion of subjects with low serum cobalamin levels and no anaemia, since fortification of the diet in the USA since 1998 and no reports in any country of an increased incidence of cobalamin neuropathy or other harmful effects. Concerns about the risk of causing or accelerating the course of cancer of any type have been allayed by the meta-analysis involving nearly 50,000 subjects discussed above. The level of fortification and the exact foods to be fortified whether whole grain, refined flour, maize or rice vary from country to country. In 2000 a UK Government Committee on Medical Aspects of Food and Nutrition Policy recommended addition of 240 µg/100 grams of flour. In 2024 the UK Government announced its intention to make addition of folic acid 250 µg/100 g to non-wholemeal flour mandatory

Folic acid before and during pregnancy

Women of child bearing age are recommended to take folic acid before and for the first three months of any pregnancy. In the UK women are divided (British National Formulary) into those considered at low risk of an NTD pregnancy and those at high risk. High risk is defined as either partner having an NTD or a family history of this or a previous NTD pregnancy or if the mother has diabetes mellitus, coeliac disease or other cause for malabsorption, is taking anti-epileptic drugs or has sickle cell anaemia. In these situations the mother is recommended to take 5 mg folic acid daily if she intends to become pregnant or is at risk of this and to continue this dose for the first 3 months of pregnancy. In sickle cell anaemia then to continue this or their normal dose of folic acid throughout the pregnancy. For the vast majority of women considered at low risk, the recommendation is to take 400 µg daily. There is no logic in this since 5 mg daily would a give them substantially greater protection against having a NTD pregnancy. There are no harmful effects of women taking folic acid 5 mg daily for weeks or a few months before becoming pregnant and for the first three months of the pregnancy. As discussed earlier there is no clear evidence that large doses of folic acid precipitate neuropathy due to cobalamin deficiency. Moreover cobalamin deficiency of sufficient severity to cause a neuropathy is also a cause of infertility. The change in

recommendation would need to be accompanied by making 5 mg tablets of folic acid available for purchase by women of child bearing age from pharmacies and not, as at present in UK, only available to them on a doctor's prescription.

Surveys have shown poor uptake of folic acid supplements by women of child-bearing age likely to become pregnant, especially in certain ethnic groups and that reinforces the need for fortification of the diet.

Prematurity

The incidence of folate deficiency is so high in the smallest premature babies during the first 6 weeks of life that folic acid (e.g. 1 mg daily) should be given routinely to babies weighing less than 1500 g at birth and to larger premature babies who require exchange transfusions or develop feeding difficulties, infections or vomiting and diarrhoea.

Haemolytic anaemia and dialysis

As discussed above folic acid is usually given to patients with a severe chronic haemolytic anaemia, for example sickle cell anaemia, or to those who are undergoing long-term haemodialysis.

Megaloblastic anaemia not due to cobalamin or folate deficiency or altered metabolism

This may occur with many antimetabolic drugs (e.g. hydroxycarbamide, cytarabine, 6-mercaptopurine) that inhibit DNA replication at a particular point in the supply of precursors or inhibit DNA polymerase. In the rare disease orotic aciduria, two consecutive enzymes in purine synthesis are defective. The condition responds to therapy with uridine, which bypasses the block. In thiamine-responsive megaloblastic anaemia, there is a genetic defect in the high-affinity thiamine transport (*SLC19A2*) gene. This causes defective RNA ribose synthesis through impaired activity of transketolase, a thiamine-dependent enzyme in the pentose cycle. This leads to reduced nucleic acid production and consequent induction of cell cycle arrest or apoptosis. It may be associated with diabetes mellitus and deafness and the presence of many ringed sideroblasts in the marrow. The biochemical explanation is unclear for megaloblastic changes in the marrow in patients with acute myeloid leukaemia, and other leukaemias and myelodysplasia in the absence of cobalamin or folate deficiency.

Other nutritional anaemias

Protein deficiency

Anaemia is usual in children and adults with severe protein deficiency (kwashiorkor). The anaemia, which may be partly masked by haemoconcentration, is usually normoblastic, but megaloblastic changes have been described in 10–60% of patients in different series. Hypoplasia, or even aplasia, of the marrow has also been reported. The mechanism by which protein deficiency causes anaemia is not completely understood. Lack of protein does not seem to reduce haemoglobin synthesis directly. Studies in experimental animals suggest that the major factor is diminution of erythropoietin secretion. This is probably due to a reduction in general tissue metabolism and therefore oxygen consumption, with a consequent reduced stimulus for erythropoietin secretion. In most patients, other factors contribute to the anaemia. These include infections, deficiencies of folate and iron, and also, possibly, deficiencies of vitamins C, E and B_{12}, and other trace substances. Riboflavin deficiency may cause anaemia in humans, resembling the anaemia of protein deficiency that it usually accompanies. The deficiency only occurs in the severely malnourished usually with other major clinical problems. Riboflavin deficiency may only become apparent during the response to protein.

Scurvy

There is usually a moderate or severe normocytic, normochromic anaemia in scurvy because of external haemorrhage and haemorrhage into tissues, and from impaired erythropoiesis. In some patients, the anaemia is megaloblastic, which appears to be partly due to associated nutritional folate deficiency and partly due to impairment of folate metabolism caused by vitamin C deficiency. However, vitamin C is not established as playing a role in normal folate metabolism.

Other deficiencies

Deficiencies of nicotinic acid and pantothenic acid cause anaemia in experimental animals but have not been shown to do so in humans. Copper is essential for haemopoiesis and normal iron metabolism; and deficiency of copper may rarely cause anaemia or more generalised marrow dysfunction. Copper excess (as in Wilson disease) causes a haemolytic anaemia.

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CHAPTER 14

Haemoglobin and the inherited disorders of globin synthesis

14

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Introduction

Inherited disorders of haemoglobin are the commonest single-gene disorders, with an estimated carrier rate of 7% amongst the world population. They occur at particularly high frequencies in populations of the tropical and subtropical belt, and consist mainly of the α- and β- thalassaemias, and the haemoglobin variants S, C and E. In many low-income countries, as economic conditions improve and infant death rates from infection and malnutrition fall, the genetic disorders of haemoglobin start to become a major burden on the health services, a phenomenon that has already been observed in many parts of the world. As a result of migrations of populations, these conditions are being seen with increasing frequency in many countries in which they had not been recognised previously.

The structure, genetic control and synthesis of haemoglobin

Different haemoglobins are synthesised in the embryo, fetus and adult, each adapted to their particular oxygen requirements (Figure 14.1). They all have a tetrameric structure made up of two different pairs (one α-like and one β-like) of globin chains, each attached to one haem molecule, the moiety responsible for the reversible binding and transfer of oxygen.

The embryonic haemoglobins include Hb Portland ($\zeta_2\gamma_2$), Hb Gower 1 ($\zeta_2\varepsilon_2$) and Hb Gower 2 ($\alpha_2\varepsilon_2$). In the fetus, HbF ($\alpha_2\gamma_2$) predominates; in adults, HbA ($\alpha_2\beta_2$) comprises over 95% of the total haemoglobin, with a minor component of HbA_2 ($\alpha_2\delta_2$) in the red blood cells. There are two kinds of HbF composed of γ-chains that differ in their amino acid composition at position 136, where they have either glycine or alanine; those with glycine are called $^{G}\gamma$-chains and those with alanine $^{A}\gamma$-chains. The $^{G}\gamma$ and $^{A}\gamma$ chains are the products of separate globin gene loci ($^{G}\gamma$ and $^{A}\gamma$). These different types of haemoglobin are adapted to the changes in physiological requirements that occur during development. Fetal haemoglobin (HbF) exhibits a higher oxygen affinity than adult haemoglobins *in vivo*; the higher oxygen affinity of HbF relative to adult haemoglobin facilitates the transfer of oxygen across the placenta from the maternal to the fetal circulation.

The sigmoid shape of the oxygen dissociation curve, which reflects the allosteric properties of haemoglobin, ensures that oxygen is rapidly taken up at the high oxygen tensions found in the lungs and is released readily at the low tensions encountered in the tissues. It is quite different from myoglobin, a molecule that consists of a single globin chain with haem attached to it and which has a hyperbolic dissociation curve. The transition from a hyperbolic to a sigmoid curve reflects cooperativity between the four haem molecules. When one haem takes on oxygen, the affinity for oxygen of the remaining haem molecules of the tetramer increases markedly. This is because haemoglobin can exist in two configurations, deoxy(T) and oxy(R) (T and R

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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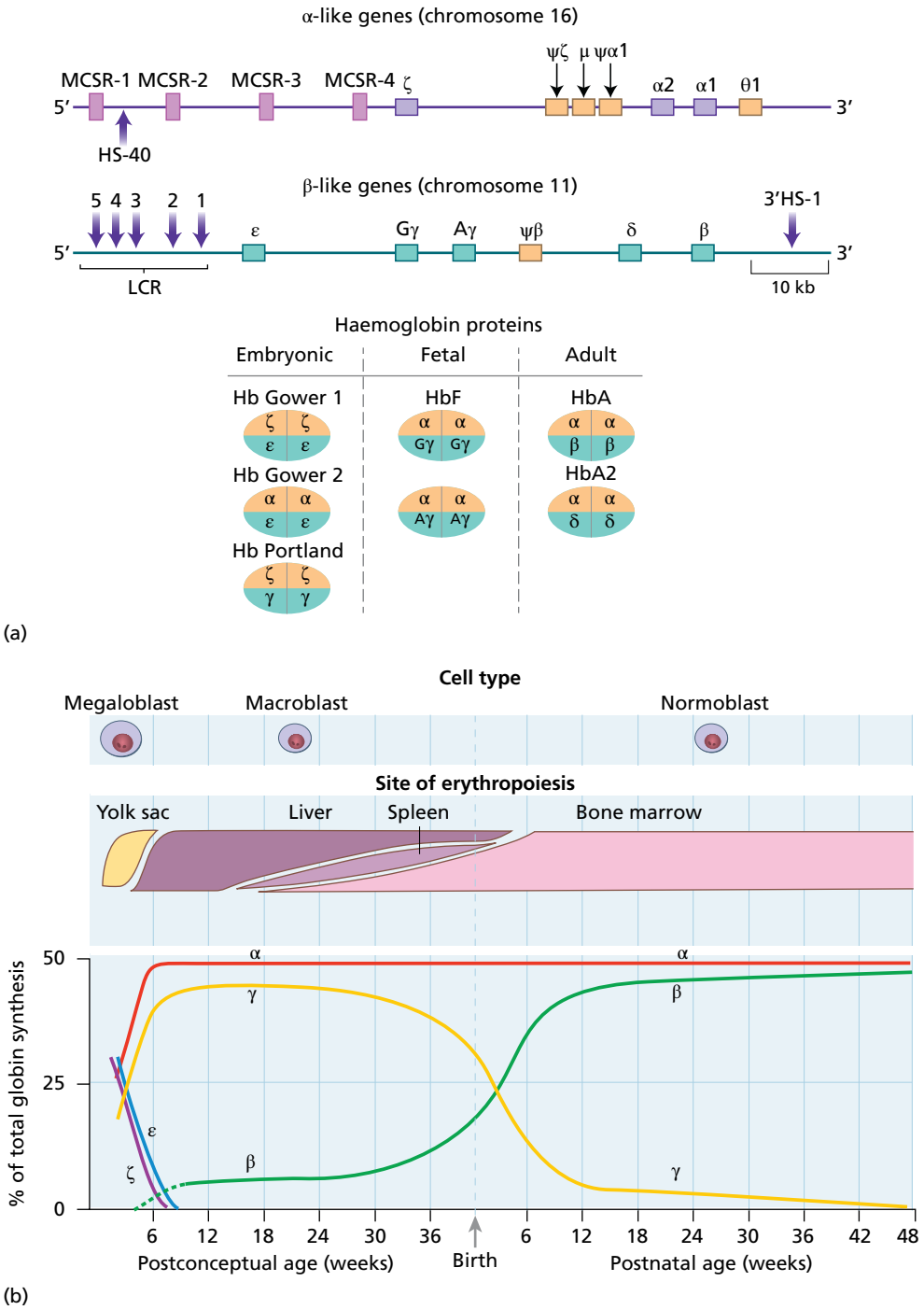

Figure 14.1 (a) The genomic structure of the clusters of α-like and β-like globin genes, on chromosomes 16 and 11, in human beings. HSs1–5 represent the set of five DNase I hypersensitivity sites distributed 5–25 kb 5′ of the ε globin gene, and which constitute the β-locus control region (β-LCR). MCSR1, R2, R3 and R4 are the four upstream erythroid-specific enhancers in the α-globin cluster. (b) The timeline of the expression of the human globin genes from early stages of fetal development to the changes that occur at birth and in the first year of life (Source: Schechter, 2008 [*Blood* 2008; **112**: 3927–38]. Reproduced with permission of the American Society of Hematology.)

stand for tight and relaxed states, respectively). The T form has a lower affinity than the R form for ligands such as oxygen. At some point during the sequential addition of oxygen to the four haems, transition from the T to R configuration occurs and the oxygen affinity of the partially liganded molecule increases dramatically. The oxygen dissociation curve, which reflects these changes, can be modified in several ways. First, oxygen affinity is decreased with increasing CO_2 concentrations – the Bohr effect. This facilitates oxygen loading to the tissues, where a drop in pH due to CO_2 influx lowers oxygen affinity. In contrast, in the lungs, efflux of CO_2 and an increase in intracellular pH increases oxygen affinity and hence uptake. Oxygen affinity is also modified by the level of 2,3-diphosphoglycerate (2,3-DPG) in the red cell. Increasing concentrations shift the oxygen dissociation curve to the right (i.e. reduce oxygen affinity), whereas diminishing concentrations have the opposite effect.

Genetic control, regulation and synthesis

Human haemoglobin production is characterised by two 'switches'. The switch from embryonic to fetal haemoglobin production begins as early as week 5 of gestation and is completed by week 10 (Figure 14.1). Expression of β-globin starts as early as week 8, but synthesis remains low, increasing to approximately 10% at weeks 30–35 of gestation, with a dramatic upregulation of β-globin synthesis just before birth, coinciding with a decrease in γ-globin expression that constitutes the fetal to adult haemoglobin switch. At birth, HbF ($\alpha_2\gamma_2$) comprises 60–80% of the total haemoglobin, falling to about 5% at 6 months of age and eventually reaching the adult level of 0.5–1.0% at 2 years. The relative synthesis of $^{G}\gamma$ and $^{A}\gamma$ chains also changes with the switch, from a $^{G}\gamma/^{A}\gamma$ ratio of 3:1 in fetal life to a ratio of 2:3 in adults. The switch from fetal to adult haemoglobin production is not total, production of variable levels of HbF persisting throughout adult life. These residual amounts are unevenly distributed; erythrocytes that contain measurable amounts of HbF are termed F cells.

Each of the α-like and β-like globin chains is encoded by genetically distinct loci, the α-like cluster on the tip of chromosome 16p and the β-like cluster on chromosome 11p15.5 (Figure 14.1). In both clusters, the genes are arranged along the chromosome in the order in which they are expressed during development: 5′ -ε-$^{G}\gamma$-$^{A}\gamma$-ψβ-δ-β-3′ and 5′ -ζ-μ-ψζ-ψα1-α2-α1-θ1-3′. The ψβ, ψζ and ψα-genes are pseudogenes, that is they have sequences that resemble the β, ζ or α-genes but contain inactivating mutations that prevent them from being expressed. They may be 'burnt out' remnants of genes that were functional at an earlier stage of evolution. Like most mammalian genes, the globin genes have one or more non-coding inserts, called intervening sequences or introns, interrupting the coding sequences or exons.

The β-like globin genes have three exons (coding regions) interrupted by two intervening sequences or introns of 122–130 and 850–900 bp, respectively. The β-genomic sequence codes for 146 amino acids; intron 1 interrupts the sequence between codons 30 and 31, and intron 2 between codons 104 and 105. The α-globin genes code for 141 amino acids and contain similar, but smaller, introns between codons 30 and 31 and between codons 99 and 100. Within each α- and β-globin complex, in addition to the primary *cis* determinants of individual globin gene expression, which are found in the immediate vicinity and within each gene, there are other local regulatory elements known as enhancers, which are located at variable distances from the individual genes.

The local *cis*-acting sequences controlling globin gene expression include the promoter region, splicing donor and acceptors and poly-A addition sites. The promoter, in the 5′ flanking region, includes blocks of nucleotide homology that are found in analogous positions in many species (Figure 14.2). The three positive *cis*-acting elements include the TATA box (position −28−−31, i.e. between 28 and 31 bases upstream from the mRNA 'cap' site), a CCAAT box (position −72−−76), and a CACCC motif which may be inverted or duplicated (position −80−−140). These promoter elements are recognised by transcription factors and are involved in the initiation of transcription. It is interesting that while the CCAAT and TATA elements are found in many eukaryotic promoters, the CACCC sequence is found predominantly in erythroid cell-specific promoters.

The 5′ untranslated region (UTR) occupies a region of about 50 nucleotides between the 5′ terminus or 'cap' site of globin mRNA and the initiation (ATG) codon. The cap appears to be important for maintaining the stability of the nuclear precursor mRNA. Within the 5′-UTR of the various globin genes there are conserved sequences that are important in the regulation of gene expression. The 3′-UTR constitutes the region between the termination codon and the poly-A tail. It consists of about 130 nucleotides with one conserved sequence, AATAAA, located 20 nucleotides upstream of the poly-A tail. The conserved hexanucleotide AATAAA acts as a signal for cleavage of the 3′ end of the primary transcript and addition of the poly-A tail, which confers stability on the processed mRNA and enhances translation. The importance of all these *cis* elements for normal globin gene expression has been validated by the discovery of thalassaemias caused by several mutations affecting these regions, as well as by deletion experiments.

Throughout development, the appropriate genes of the α- and β-globin gene clusters are coordinately expressed, maintaining a balance in the production of α- and β-like globins needed for the synthesis of normal haemoglobin. The regulation of globin gene expression is mediated at several levels; although most occur at the transcriptional level, there is some fine-tuning during and after translation. Most DNA that is not involved in gene transcription is tightly packaged into a compact, chemically modified form that is inaccessible to transcription factors and polymerases and which is heavily methylated. Activity is associated with a change in the structure of the chromatin surrounding a gene, which can be identified

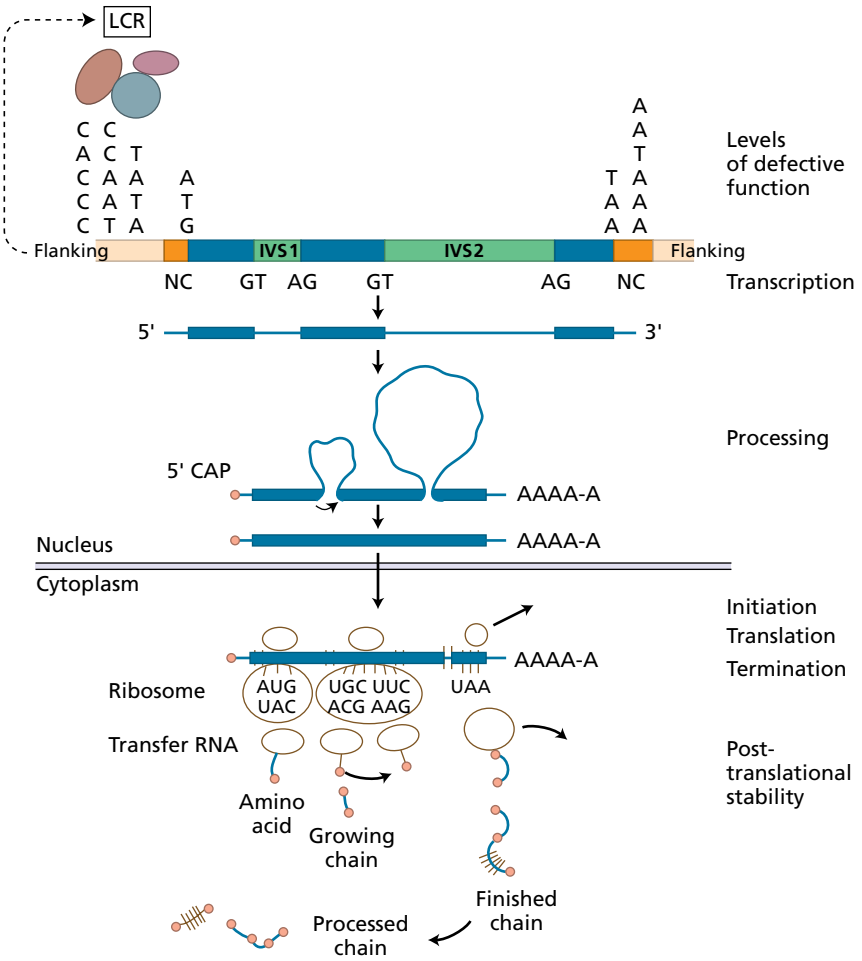

Figure 14.2 A prototype globin gene and the genetic control of globin chain synthesis. Levels of action of mutations are indicated on the right.

by enhanced sensitivity to nucleases. Erythroid lineage-specific nuclease hypersensitivity sites are found at several locations in both the β-globin and α-globin gene clusters. A set of five DNase I hypersensitivity sites (designated HSs1–5), distributed 5–25 kb 5′ of the ε globin gene, constitute the β-locus control region (β-LCR) (see Figure 14.1). This region was originally implicated in the control of the β-globin complex by the discovery of natural mutants that removed sequences upstream but left the downstream globin genes intact and yet resulted in no output from the cluster. The human β-LCR was the first LCR to be identified and was functionally defined as a DNA element that provides high levels of tissue-specific expression to a *cis*-linked gene in a copy number-dependent manner, and which is independent of host genome integration site. The corresponding region in the α-globin cluster consists of four multispecies conserved sequence (MCS) regions lying 10–48 kb upstream of the α-globin genes called MCS-R1 to MCS-R4: 10 kb (MCS-R4), 33 kb (MCS-R3), 40 kb (MCS-R2) and 48 kb (MCS-R1) upstream (see Figure 14.1). The four upstream erythroid-specific enhancers vary in their capacity to enhance α globin expression but MCS-R2, which consists of a single DNase hypersensitive site, appears to be the most critical. MCS-R2, which lies 40 kb upstream of the cluster, is previously known as HS-40. The β-LCR establishes a transcriptionally active chromatin domain that encompasses the whole β-globin cluster and acts as a unique enhancer, whereas the α-globin MCS-R2 which is most similar to HS2 of the β-LCR is capable of enhancing α globin expression on its own. Importance of the MCS-R2 is further confirmed by the naturally occurring upstream human deletions that downregulate α globin expression, all involve the MCS-R2 site. In both clusters, expression of the respective genes is critically dependent on the presence of the upstream regulatory elements that physically interact with upstream promoters of the respective globin gene mediated by a complex of transcription factors.

Several other enhancer sequences have also been identified in both globin gene clusters. All these regulatory regions bind a number of key erythroid-specific transcription factors, notably GATA-1, GATA-2, NF-E2, KLF1 (also known as EKLF) and SCL, as well as various cofactors (e.g. FOG, pCAF and p300) and factors that are more ubiquitous in their tissue distribution, such as Sp1. Tissue-specific expression may be explained by the presence of

binding sites for the erythroid-specific transcription factors. The binding of haemopoietic-specific factors activates the LCR, which renders the entire β-globin gene cluster transcriptionally active. Transcription factors also bind to enhancer and local promoter sequences within each gene, which work in tandem to regulate the expression of the individual genes in the clusters. Some of the transcription factors are developmental-stage specific and may be involved in the (still poorly understood) differential expression of embryonic, fetal and adult globin genes.

The mechanisms by which developmental regulation is controlled have become clearer in the last decade, building on the proposed dual mechanism of autonomous gene silencing and gene competition for direct interaction with the upstream LCR. It appears that the ε and ζ genes are switched on in embryonic cells and autonomously switched off (transcriptionally silenced) in definitive cells (liver and bone marrow) in which they cannot be substantially reactivated. The second switch, from γ- to β-gene expression, is more complex and involves both autonomous silencing of the γ-genes and competition between the γ- and β-genes for the β-LCR. In the γ- to β-gene switch, the γ globin genes are autonomously silenced at the appropriate developmental stage, and expression of the adult β globin gene depends on lack of competition from the upstream γ gene for the LCR sequences. In accordance, when 'silencing' of the γ gene fails due to point mutations in their promoter causing a persistence of fetal haemoglobin production, expression of the *cis* β gene is downregulated. The transcriptional factor environment is thus critical in determining the balance between γ- and β-gene expression and is thought to be mediated by changes in the repertoire and/or abundance of various nuclear factors favouring particular promoter–LCR interactions. The erythroid Krüppel-like factor (*EKLF*, also known as *KLF1*) was the first example of a developmental stage-specific regulatory factor without which the β-genes cannot be fully activated in the definitive cells. Not only is KLF1 expression restricted mainly to erythroid cells, but it is also a highly promoter-specific activator, binding with high affinity to the β-globin CACCC box. Its greater affinity for the β-globin than the γ-globin promoter accelerates the shutdown of γ in transgenic mice over-expressing klf1. Genome wide association (GWA) studies identified BCL11A as the first key repressor protein for silencing of the γ globin genes (joined later by ZBTB7A/LRF). KLF1 is a direct activator of *BCL11A*. KLF1 is key in the switch from γ-globin to β-globin expression; it not only activates the β-globin gene directly, providing a competitive edge, but also silences the γ-globin genes indirectly via activation of *BCL11A*. A genetic network regulating the switch from γ- to β-globin expression has emerged, involving the interaction of KLF1, BCL11A, LRF and MYB with each other, and other transcription factors (e.g. GATA-1) and corepressor complexes that involve chromatin modelling and epigenetic modifiers. KLF1 may also play a role in the silencing of the embryonic globin genes. KLF1 has emerged as a major erythroid transcription factor with pleiotropic roles underlying many of the previously uncharacterised anaemias (e.g. congenital dyserythropoietic anaemia type IV). KLF1 variants have also been associated with variable increases in HbF and HbA_2 levels.

It is still not clear how KLF1 interacts with LRF/ZBTB7A, the other major γ globin repressor. High-resolution studies of BCL11A and LRF in the *HBB* cluster by two groups showed that BCL11A and LRF each bind to a cognate recognition site within the γ-globin promoter. The repressive activity of both BCL11A and LRF is mediated through physical interaction with the multicomponent nucleosome remodelling and deacetylase (NuRD) complex. The important role of the NuRD complex in silencing of the γ-globin genes is provided by recent findings that repression of the NuRD subunit CHD4 expression by ZNF410 increases HbF expression.

The binding sites of LRF and BCL11A coincides with positions of the base substitutions observed in subjects with hereditary persistence of fetal haemoglobin (HPFH). Thus, one approach to increasing γ-globin expression could involve reducing expression of the repressors BCL11A or LRF, or to disable binding of BCL11A or LRF by genome-editing, i.e. mimicking the naturally occurring HPFH variants in the γ-globin promoters. Besides its role as γ-globin repressor, BCL11A has pleiotropic roles in B-lymphoid and neurological development. Identification of the key erythroid-specific enhancer elements was critical and important in the development of the clinical trials aimed at downregulating BCL11A for therapeutic activation of fetal haemoglobin.

Transcription and processing of mRNA

Genetic information in the DNA of the globin genes is transcribed into an RNA copy which is then translated into a specific globin chain (Figure 14.2). The TATA box acts as the initial DNA target for the progressive assembly of an initial transcription complex, which involves the interaction of transcription factors, TATA-binding protein and other proteins with the β-LCR or α-MCS, mediated by RNA polymerase II.

The primary transcript is a large mRNA precursor (pre-mRNA) that contains both introns and exons. While in the nucleus, it undergoes a number of modifications before it can be translated into protein (Figure 14.2). This includes the removal of introns by a complex series of reactions involving several different proteins that constitute the spliceosome, and splicing of the exons. Consensus sequences are universally found encompassing the 5′ (donor) and 3′ (acceptor) ends. Each intron invariantly starts with the dinucleotide GT (5′) and finishes with AG (3′). Mutations that alter the normal consensus sequences or mutations that create similar consensus sequences at new sites in globin genes cause aberrant splicing and constitute the molecular basis of many types of thalassaemia. Other modifications of the nascent mRNAs include the addition of a 'cap' structure at the 5′ end and the addition of a string of

adenylic acid residues (poly-A) at the 3′ end. Proper cleavage of the primary RNA transcript and polyadenylation of the 3′ ends of mRNA is guided by a consensus hexanucleotide (AATAAA) sequence about 20 nucleotides upstream of the poly-A tail. The processed mRNA now moves into the cytoplasm to act as a template for globin chain production on defined organelles known as ribosomes.

Translation

Amino acids are transported to the mRNA template on carriers called transfer RNAs; there are specific transfer RNAs for each amino acid. The order of amino acids in a globin chain is determined by the order of nucleotides (reading frame); three bases (codon) code for a particular amino acid. The transfer RNAs also contain three bases, the anticodon, which are complementary to mRNA codons for particular amino acids. The transfer RNAs carry amino acids to the template, where they find the right position by codon–anticodon base-pairing. The mRNA is translated from the 5′ to the 3′ end (left to right) starting with a specific initiation codon (AUG) and ending with a termination codon (UAA, UAG, UGA). When the ribosome reaches the termination codon, translation ceases, the completed globin chain is released, and the ribosomal subunits fall apart and are recycled. Individual globin chains combine with haem, which is synthesised through a separate pathway, and with themselves to form definitive haemoglobin molecules.

Termination codons are also called nonsense codons because they do not usually encode any amino acid. Approximately 50% of the mutations causing β-thalassaemia are caused by termination codons that are premature. Premature termination codons (PTCs) can result from different types of mutations. Single nucleotide substitutions can convert a sense codon to a nonsense codon and are often referred to as nonsense mutations. Frameshift mutations are insertions or deletions of a few bases that are not multiples of three that shift the reading frame, resulting in a nonsense codon that is premature (i.e. PTC). mRNAs with PTCs lead to production of encoded truncated proteins that are potentially harmful. They are kept in check by a cellular surveillance mechanism referred to as nonsense-mediated mRNA decay (NMD). NMD is usually triggered when translation stops prematurely at PTCs, resulting in termination of the mutant mRNA transcript and thus absence of abnormal protein. However, PTCs situated less than 50–55 nucleotides upstream of the 3′ -most exon–exon junction or downstream of this junction generally fail to trigger NMD, and result in production of abnormal mRNA species. In the case of the β-globin gene, PTCs within the last exon (exon 3) and 3′ half of exon 2 result in the production of highly unstable β-globin variant chains and a dominantly inherited form of β-thalassaemia. However, there are exceptions to the 50–55 rule; PTCs within β-globin exon 1 and a PTC within exon 2 have been reported to fail to elicit NMD efficiently despite residing more than 55 nucleotides upstream of the exon 2–exon 3 junction.

The multistep process in the conversion of DNA into protein offers numerous opportunities for mishaps to occur that result in downregulation of gene expression, clearly illustrated by the different mutations causing thalassaemias and haemoglobinopathies.

Classification of the disorders of haemoglobin

Mutations in the globin genes can cause a quantitative reduction in output from that gene, alter the amino acid sequence of the protein produced or a combination of the two (Table 14.1). Quantitative defects cause thalassaemia syndromes, whereas qualitative changes referred to as haemoglobin variants, cause a wide range of problems, including sickle cell disease (Chapter 15), unstable haemoglobins, decreased oxygen affinity, increased oxygen affinity and methaemoglobinaemia. However, the majority of qualitative mutations cause no significant change in haemoglobin properties or clinical problems. Some mutations combine both features, resulting in a haemoglobin variant that is made in reduced amounts; HbE (β26 Glu→Lys) is the most common example of this. The substitution at codon 26 (GAG→AAG), which causes HbE, also causes alternative splicing of the β-globin mRNA, leading to a reduction in the normally spliced β-message encoding the variant, and a

Table 14.1 The thalassaemias and related disorders.

β-Thalassaemia
β^0
Deletion
Non-deletion
β^+
'Silent'
Normal HbA_2
Dominant
α-Thalassaemia
α^0
α^+
Deletion (/−α)
Non-deletion (/α^T α)
δβ-Thalassaemia
$^G\gamma^A\gamma\ (\delta\beta)^0$
$^G\gamma\ (^A\gamma\delta\beta)^0$
$(\delta\beta)^+$
γ-Thalassaemia
δ-Thalassaemia
εγδβ-Thalassaemia
Hereditary persistence of fetal haemoglobin
Deletion
Non-deletion
$^A\gamma$
$^G\gamma$

thalassaemia phenotype. Other haemoglobin variants result in a thalassaemia phenotype caused by extreme instability and functional deficiency of the globin chain variant; for example, Hb Geneva, a dominantly inherited β-thalassaemia. Mutations in the globin gene complex might also alter the switch from fetal to adult haemoglobin synthesis, resulting in persistence of γ-globin expression, and hereditary persistence of fetal haemoglobin (HPFH).

The thalassaemias and related disorders

The thalassaemias are the most common single-gene disorders. Thalassaemia was first recognised by Cooley and Lee in 1925 as a form of severe anaemia associated with splenomegaly and bone changes in children. The term 'thalassaemia' is derived from the Greek θαλασσα-(meaning 'the sea') since many of the early cases came from the Mediterranean region. However, it is now clear that the disorder is not just limited to the Mediterranean region, but occurs throughout the world, prevalent in the tropical and subtropical regions, including the Middle East, parts of Africa, Indian subcontinent and Southeast Asia. Heterozygotes for thalassaemia are protected from the severe effects of malaria and natural selection has increased and maintained their gene frequencies in these malarious regions.

Definition and classification

The thalassaemias are classified into α-, β-, δβ-, γδβ-, δ-, γ- and εγδβ-thalassaemias, according to the type of globin chain(s) that is produced in reduced amounts (Table 14.1). The two major categories are the α- and β-thalassaemias, while the rare forms include the γ-, δ- and εγδβ- thalassaemias.

Functionally, some thalassaemia mutations cause a complete absence of globin chain synthesis, and these are called α^0 or β^0-thalassaemias; in others, the globin chain is produced at a reduced rate and these are designated α^+ or β^+-thalassaemias. The δβ-thalassaemias are subdivided in the same way. HPFH syndromes refer to the group of disorders in which the switch from fetal to adult haemoglobin production is incomplete and variable levels of fetal haemoglobin levels persist in otherwise normal individuals. Because of their concomitant increased HbF levels, the δβ- and γδβ-thalassaemias are often considered with the HPFH syndromes.

Because thalassaemia occurs in populations in which structural haemoglobin variants are common, it is not unusual to inherit a thalassaemia gene from one parent and a gene for a structural haemoglobin variant from the other. Furthermore, both α- and β-thalassaemia occur commonly in some countries, and individuals may coinherit genes for both types. These different interactions produce a clinically diverse family of genetic disorders that range in severity from death *in utero* to extremely mild, symptomless and hypochromic anaemias.

Most thalassaemias are inherited in a Mendelian recessive fashion. Heterozygotes are mostly symptomless, although usually they can be recognised by simple haematological analysis. More severely affected patients are either homozygotes for α- or β-thalassaemia or compound heterozygotes for different molecular forms of α- or β-thalassaemia or for one or other form of thalassaemia and a gene for a haemoglobin variant. Clinically, the thalassaemias are classified according to their severity into major, intermediate and minor forms. Thalassaemia major is a severe and transfusion-dependent disorder. Thalassaemia minor is the symptomless trait or carrier state. Thalassaemia intermedia is characterised by anaemia (with or without splenomegaly), though not of such severity as to require regular transfusion. In practice, thalassaemia intermedia encompasses a wide spectrum of clinical severities intermediate between the two extremes of thalassaemia major and trait. Thalassaemia intermedia, also referred to as non-transfusion-dependent thalassaemia (NTDT) remains a clinical definition and includes β-thalassaemia intermedia, HbH disease and the HbE/β-thalassaemias.

The β-thalassaemias

The β-thalassaemias pose by far the most important public health problems because they are common and usually produce severe anaemia in their homozygous and compound heterozygous states.

Distribution

The β-thalassaemias occur widely in a broad belt, ranging from the Mediterranean and parts of North and West Africa through the Middle East and Indian subcontinent to Southeast Asia. The disease is particularly common in Southeast Asia, where it occurs in a line starting in southern China and stretching down through Thailand and the Malay Peninsula and Indonesia to some of the Pacific Island populations. In this region, and in some of the Mediterranean island and mainland countries, gene frequencies range between 2 and 30%. It should be remembered that β-thalassaemia is not confined entirely to these high-incidence regions and it occurs sporadically in every racial group.

Genetic basis of disease: molecular pathology

The β-thalassaemias are considered to be autosomal recessive disorders since individuals who have inherited one abnormal β-gene (carrier) are asymptomatic and the inheritance of two abnormal β-globin genes is required to produce a clinically detectable phenotype. Molecular analysis of the β-thalassaemia genes has demonstrated a striking heterogeneity. Although almost 300 β-thalassaemia alleles (including deletions) have been characterised, population studies indicate that probably only 20 β-thalassaemia alleles account for more than 80% of the

β-thalassaemia mutations in the whole world (https://www.ithanet.eu; http://globin.bx.psu.edu/hbvar). This is because in each of the high-frequency areas, only a few (four to six) mutations are common, reflecting local selection due to malaria, with a varying number of rare ones. Each of these populations thus has its own unique group of mutations.

The vast majority (approximately 250) of β-thalassaemia mutations are point mutations (i.e. single-base substitutions) and small insertions or deletions of one to two bases. These may involve any step in globin chain production: transcription, translation or post-translational stability of the globin gene product (Figure 14.2). Approximately half of these mutations completely inactivate the β-gene with no β-globin production resulting in β^0 -thalassaemia. Mutations that allow the production of some β-globin cause β^+ – or β^{++} -thalassaemia, depending on whether there is a marked or mild reduction in the output of β-chains, respectively.

Transcription

The mutations that interfere with transcription include deletions and point mutations involving the globin gene promoter regions. With the exception of a deletion of about 600 bases at the 3′ end of the β-globin gene, which is restricted to certain Indian populations, major deletions are uncommon. A large number of point mutations involve the promoters or adjacent regions, most of which downregulate the β-globin gene to a variable degree and cause relatively mild forms of β-thalassaemia.

A couple of β-thalassaemia mutations in this class are 'silent' i.e. carriers who do not have any evident haematological phenotypes, with red cell indices and HbA_2 levels within the normal range, the only abnormality being imbalanced globin chain synthesis. These β-thalassaemia mutations have usually been 'discovered' in individuals with thalassaemia intermedia resulting from compound heterozygosity for one of those 'silent' mutations in combination with a typical β-thalassaemia mutation. In this case, one parent has typical β-thalassaemia trait and the other is apparently normal. Overall, the 'silent' β-thalassaemia alleles are uncommon, except for the −101 C→T mutation that has been observed fairly frequently in the Mediterranean region, where it interacts with a variety of more severe β-thalassaemia mutations to produce milder forms of β-thalassaemia intermedia.

Processing

A wide variety of mutations interfere with processing of the primary mRNA transcript. Those involving the invariant GT or AG sequences at intron–exon junctions prevent splicing altogether and cause β^0-thalassaemia. Mutations involving the consensus sequences adjacent to the GT or AG dinucleotides in the introns allow some normal splicing and causes β^+-thalassaemia. Several β-thalassaemia mutations involve other parts of the introns; alternative splicing sites are produced leading to variable degrees of both normal and abnormal mRNA synthesis. An incorrectly spliced mRNA is not functional because it contains intron sequences, generating a frameshift and a PTC. Sequences that resemble the consensus sequences at intron–exon junctions are also present in exons. Mutations may activate these 'cryptic' sites, again leading to abnormal splicing.

Translation

About half of the β-thalassaemia alleles completely inactivate the gene, mostly by generating PTCs, either by single-base substitution to a nonsense codon or through a frameshift mutation. As part of the surveillance mechanism that is active in quality control of the processed mRNA, mRNA harbouring a PTC is destroyed and not transported to the cytoplasm (a phenomenon called nonsense-mediated mRNA decay, NMD) to prevent the accumulation of mutant mRNAs coding for truncated peptides. However, some in-phase PTCs that occur later in the β-sequence, in the 3′ half of exon 2 and in exon 3, escape NMD and are associated with substantial amounts of mutant β-mRNA, leading to synthesis of β-chain variants that are highly unstable and non-functional with a dominant negative effect (see next section). Other mutations of RNA translation involve the initiation (ATG) codon. Nine of these have been described; apart from an insertion of 45 bp, all are single base substitutions and again result in β^0 -thalassaemia.

Mutations affecting post-translational stability

Instability of the β-globin gene product is the basis for the dominantly inherited β-thalassaemias (Figure 14.3). As discussed earlier, in-phase PTCs within the 3′ half of exon 2 and in exon 3 cannot efficiently elicit NMD and hence abnormal mRNA containing the PTCs are transported to the cytoplasm and translated. However, these truncated variant β-chains are highly unstable, non-functional and not able to form viable tetramers. They precipitate in the erythroid precursors together with the redundant α-chains, causing premature death of these cells, and accentuating the ineffective erythropoiesis. Severe anaemia and clinical disease result even in the heterozygous state. Highly unstable β-globin chains can also result from single-base substitutions or minor insertions/deletions that affect a critical amino acid of the β-globin peptide that is involved in α/β dimer formation or haem binding. In other cases, the minor insertions/deletions lead to shifts in the reading frame resulting in long unstable β-globin gene products that form prominent inclusion bodies in red cell precursors.

Deletions restricted to the β-globin gene

β-Thalassaemia is rarely caused by deletions (Figure 14.4). Of these, only the 619-bp deletion at the 3′ end of the β-gene is common, but even that is restricted to the Sind populations of

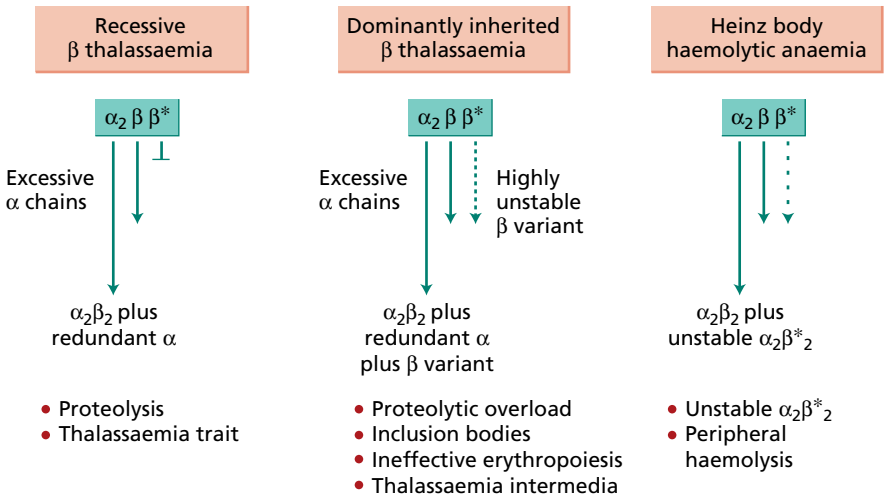

Figure 14.3 Heterozygous mutations in the β-globin gene and the different phenotypes.

India and Pakistan where it constitutes about 30% of the β-thalassaemia alleles. The other deletions, although extremely rare, are of particular clinical interest because they are associated with unusually high levels of HbA_2 and HbF in heterozygotes. The increase in HbF is adequate to compensate for the complete absence of HbA in homozygotes for these deletions. The mechanism underlying the elevated levels of HbA_2 and HbF appears to be related to removal of the 5′ promoter region of the β-globin gene, which removes competition for the upstream β-LCR and limiting transcription factors, resulting in increased interaction of the LCR with the γ- and δ-genes in *cis*, thus enhancing their expression. This mechanism may also explain the unusually high HbA_2 levels that accompany the point mutations in the β-promoter region.

Unusual causes of β-thalassaemia

These are extremely rare and are mentioned here not just for the sake of completeness but also to illustrate the numerous molecular mechanisms that downregulate the β-globin gene.

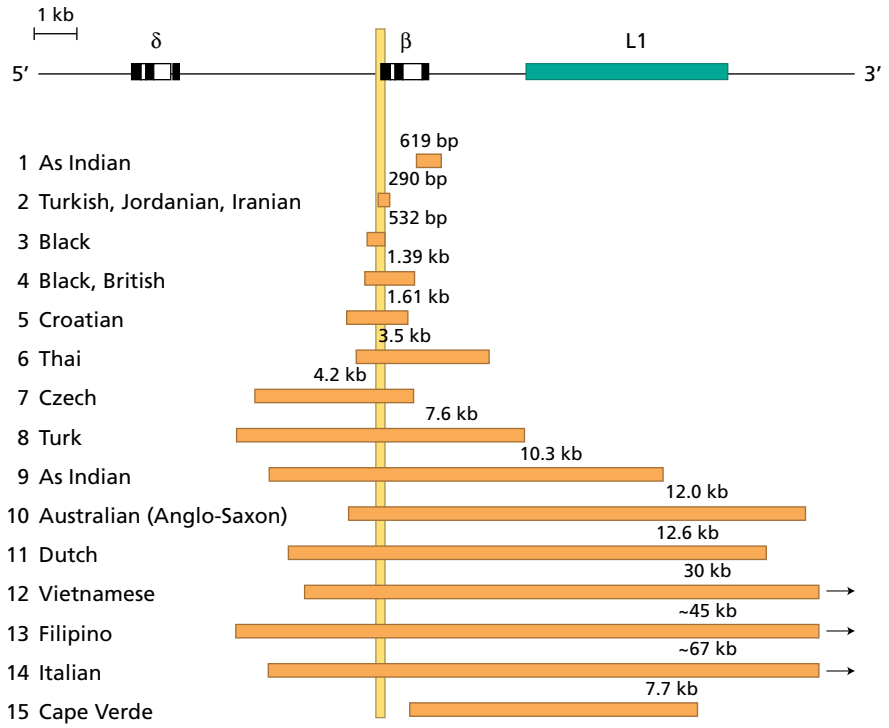

Figure 14.4 Deletions causing β-thalassaemia. The vertical bar indicates the β-globin promoter region that is removed in common by these deletions, except for the 619 bp deletion. The horizontal arrows indicate that the 3′ end of the deletions have not been defined.

Transposable elements may occasionally disrupt human genes and result in their inactivation. The insertion of such an element, a retrotransposon of the LI family, into intron 2 of the β-globin gene has been reported to cause β^+-thalassaemia. Rarely, mutations in other genes distinct from the β-globin complex can downregulate β-globin expression. Such *trans*-acting mutations have been described, affecting the XPD protein, which is part of the general transcription factor TF11H, and the key erythroid-specific transcription factor GATA-1. Somatic deletion of the β-globin gene contributed to thalassaemia intermedia in three unrelated families of French and Italian origins. The affected individuals with thalassaemia intermedia were constitutionally heterozygous for β^0-thalassaemia, but subsequent investigations revealed a somatic deletion of chromosome 11p15, including the β-globin gene complex, in *trans* to the mutation in a subpopulation of erythroid cells. This results in a somatic mosaic: 10–20% of the cells were heterozygous with one normal copy of the β-globin gene, and the rest hemizygous (i.e. without any normal β-globin gene). Unusually severe anaemia can also result from uniparental isodisomy of chromosome 11p, which encompasses the β-globin gene complex. In one case, thalassaemia major in a Chinese patient was caused by homozygosity for a paternal β-thalassaemia allele due to unipaternal isodisomy of chromosome 11p15.5, and in another, a previously healthy Portuguese adolescent carrier for β-thalassaemia became transfusion-dependent due to mosaicism of chromosome 11p14.3-11p15 that contained a β^0 CD15 mutation.

Pathophysiology

The molecular defects in β-thalassaemia result in absent or reduced β-chain production while α-globin synthesis is unaffected, resulting in an imbalance in globin chain production and an excess of α-chains. The free α-globin chains are the central causative factor in the cellular pathology and pathophysiology of β-thalassaemia. The unchaperoned α-globin chains are highly unstable and precipitate in red cell precursors, forming intracellular inclusions that interfere with red cell maturation (Figure 14.5). There is a variable degree of intramedullary destruction of erythroid precursors (i.e. ineffective erythropoiesis) that characterises all β-thalassaemias. Those red cells that mature and enter the circulation contain α-chain inclusions that interfere with their passage through the microcirculation, particularly in the spleen. However, the damage to red cell precursors and their progeny in β-thalassaemia is not entirely mechanical. The degradation products of excess α-chains, particularly haem and iron, produce a wide range of deleterious effects on red cell membrane proteins and lipids, manifested by marked abnormalities of electrolyte homeostasis and membrane deformability. The end result is an extremely rigid red cell with a shortened survival.

Thus, the anaemia of β-thalassaemia results from a combination of ineffective erythropoiesis and haemolysis. The anaemia stimulates erythropoietin production, which causes expansion of the bone marrow and may lead to serious deformities of the skull and long bones. Because the spleen is being constantly

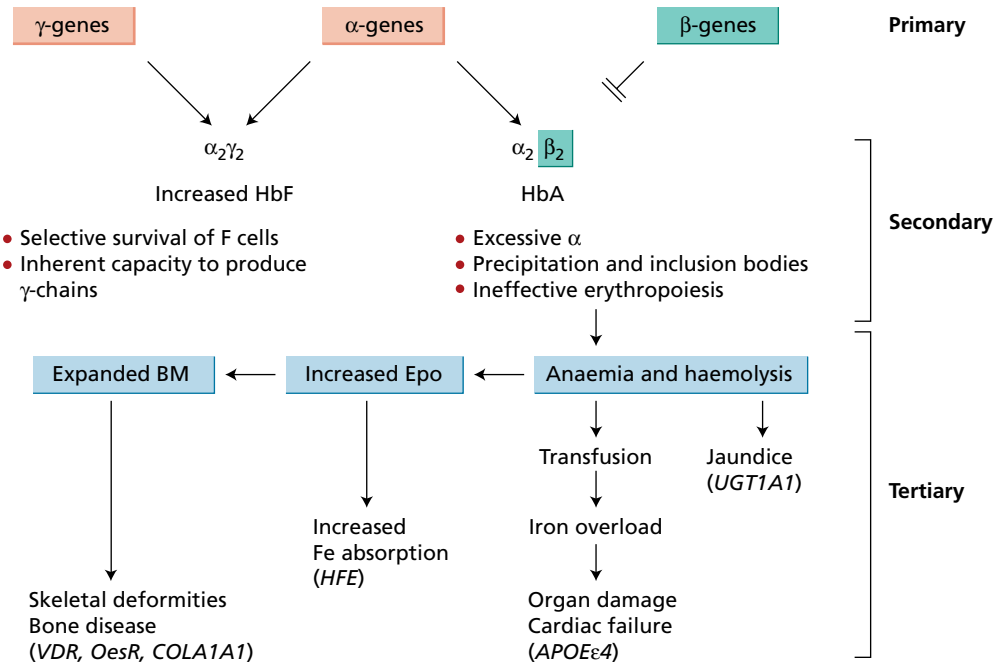

Figure 14.5 The pathophysiology of β-thalassaemia. BM, bone marrow; Epo, erythropoietin (Source: Thein, 2004 [*Br J Haematol* **124**: 264–74]. Reproduced with permission of Wiley.)

bombarded with abnormal red cells, it hypertrophies. The resulting splenomegaly, together with bone marrow expansion, causes a major increase in plasma volume, which also contributes to the anaemia.

As mentioned previously, HbF production almost ceases after birth. However, some adult red cell precursors retain the ability to produce a variable amount of γ-chains. Because the latter can combine with excess α-chains to form HbF, cells that make relatively more γ-chains in the bone marrow are partly protected against the deleterious effect of α-chain precipitation. These F cells come under selection in the marrow and peripheral blood and thus individuals with β-thalassaemia have variable increases in HbF due to selective survival of these F cells. In some cases, there is also a genuine increase in HbF production, as well as selection of F cells due to coinheritance of genetic determinant(s), or quantitative trait loci (QTL), which increase HbF production.

It follows therefore that if the anaemia is corrected with blood transfusion, the erythropoietin drive is shut off, growth and development are normal, bone deformities do not occur and splenomegaly is less marked. On the other hand, each unit of blood contains 200–250 mg of iron, and with regular transfusion there is steady accumulation of iron in the liver, endocrine glands and myocardium. Thus, although well-transfused thalassaemic children grow and develop normally, they die of iron overload unless steps are taken to remove iron.

Genotype–phenotype relationships

The β-thalassaemias show remarkable phenotypic variability, ranging from severe life-threatening anaemia to an extremely mild condition that may be identified only by chance. The molecular basis for this diversity is at least partly understood.

The genetic modifiers of the β-thalassaemia phenotype can be divided into primary, secondary and tertiary (Figure 14.5). Primary modifiers are the different mutations that affect the β-globin gene. These have variable effects on β-globin gene expression that may affect the output of β-globin chains, ranging from zero to a very mild reduction. Secondary modifiers are those that reduce the degree of imbalance of the globin chains. They include the coinheritance of α-thalassaemia and a variety of genetic modifiers of γ-chain production in adult life. So far, three major QTLs – *Xmn*1-^{G}γ site, *HBS1L-MYB* intergenic polymorphisms (*HMIP*) on chromosome 6q, and *BCL11A* gene on chromosome 2 – have been mapped. It seems likely that the genetic variance that remains unaccounted for may be due to many common and/or rare alleles with small effects on the trait, i.e. a polygenic component.

Coinheritance of α-thalassaemia reduces chain imbalance and disease severity in individuals who have inherited two copies of β-thalassaemia alleles, while the increased output of α-globin through coinheritance of extra α-globin genes in β-thalassaemia heterozygotes increases chain imbalance, converting a typically asymptomatic state to that of thalassaemia intermedia. The outcome depends on the number of α-globin genes inherited as one or two copies of triplicated (/ααα) or quadruplicated (/αααα) α-globin complexes, and the type of β-thalassaemia mutation (β^0 or β^+). A rarer mechanism of inheriting extra α-globin genes involves segmental duplication of the whole α-globin gene cluster.

Tertiary modifiers are those that affect the complications of disease; the severity of bone disease, iron loading and jaundice may be affected by polymorphisms of genes involved in the metabolic pathways concerned with these complications. Bone mass, like HbF, is a quantitative trait under strong genetic control involving multiple QTLs, those implicated including oestrogen receptor gene, vitamin D receptor (*VDR*) gene, collagen type α1 genes and transforming growth factor β1 (*TGFB1*) gene. Studies have shown that the levels of bilirubin and incidence of gallstones are related to a polymorphic variant (seven TA repeats) in the promoter of the uridine diphosphate glucuronosyltransferase 1A (*UGT1A1*) gene, also referred to as Gilbert syndrome. Iron loading in β-thalassaemia results not just from blood transfusion but also from increased iron absorption. Variants in the *HFE* gene have a modulating effect on iron absorption, and as other genes in iron homeostasis become uncovered, it is likely that there will be genetic variants in these loci that influence the different degrees of iron loading in β-thalassaemia. Similarly, it seems very likely that the propensity to infection is modified by polymorphisms involving the immune system and its regulation. Finally, it should be remembered that environmental factors, long neglected, may also play an important role in modifying the β-thalassaemic phenotype.

Clinical findings in severe β-thalassaemia

In many high-income countries, neonatal screening programmes will identify babies with more severe forms of β-thalassaemia, before the development of any symptoms; in some cases, antenatal screening of the parents and possibly prenatal diagnosis will have identified the fetus to be at high risk of β-thalassaemia before birth. Mutations in the β-globin gene almost never cause clinical symptoms *in utero* or neonatally due to the predominance of γ-globin at this stage. However, in many countries, neonatal screening programmes do not exist and diagnosis in the child will depend on their symptomatic presentation. Severe β-thalassaemia usually presents in the first year of life. Typically, there is failure to thrive, with poor weight gain and growth with developmental delay. The parents may have noticed that the infant is pale and jaundiced, with a protruding abdomen. There may be a family history of severe anaemia, and typically the family will not be of northern European origin. Examination confirms the pallor and jaundice, with palpable hepatosplenomegaly. There may be evidence of marked erythroid hyperplasia, with typical 'thalassaemic facies' including expansion of the skull vault and maxillary bones. The symptoms and signs are not specific

and differential diagnoses include gastrointestinal or hepatic disease, chronic infection and malignancy.

Laboratory diagnosis of severe β-thalassaemia

In untransfused patients with severe β-thalassaemia, the full blood count shows marked anaemia with the haemoglobin usually less than 50 g/L. Mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) are low, with a very wide red cell distribution width. The nucleated cell count may be very high due to the presence of large numbers of nucleated red cells. A blood film shows marked anisopoikilocytosis, with basophilic stippling and small red cell fragments (Figure 14.6). The reticulocyte count is elevated, but less than expected for the degree of anaemia, due to the ineffective erythropoiesis. Renal function is normal, but liver function tests show elevation of bilirubin, aspartate aminotransferase and lactate dehydrogenase, with a normal alanine aminotransferase. Erythropoietin levels will be high, with soluble transferrin receptor levels up to 30 times greater than normal, reflecting marked expansion of the erythron. White cell and platelet counts should be normal unless there is hypersplenism. A bone marrow aspirate is not essential to make the diagnosis, but if performed shows very marked erythroid hyperplasia, with dyserythropoiesis. Many of the erythroid precursors show inclusions after incubation with methyl violet; similar inclusions are found in the peripheral red cells after splenectomy. Immunoelectron microscopy confirms that the inclusions in β-thalassaemia consist of precipitated α-globin chains.

Haemoglobin analysis is needed to confirm the diagnosis, typically using either electrophoretic or chromatographic techniques. This will usually show an increased amount of HbA_2, with the vast majority of the remainder consisting of HbF; small amounts of HbA may be present depending on the β-globin mutation, the age of the child and whether the child has been transfused. The absence of HbA confirms a diagnosis of β^0 thalassaemia, while presence of HbA (pretransfusion sample) confirms β^+-thalassaemia. Testing of the parents should confirm the diagnosis, both typically being carriers of β-thalassaemia, with HbA_2 levels greater than 3.5% and MCH below 27 pg. These findings are sufficient to make a diagnosis of severe β thalassaemia, although where DNA analysis is available it is often used to identify the β-globin mutations and confirm the diagnosis.

Management of severe β-thalassaemia

Any child presenting in the first year of life with the features described above is likely to require regular red cell transfusions to grow and develop normally; this is referred to as thalassaemia major or transfusion-dependent thalassaemia. If such children are not transfused regularly, as happened historically, and as happens currently in many low-income countries, progressive deterioration occurs. Growth and development continue to be severely impaired. The child often has muscle wasting due to increased metabolic demands, and in particular, may become folate deficient. The spleen and liver become progressively enlarged; the spleen can become massive with hypersplenism and resulting cytopenias. There is marked erythroid hyperplasia with bony distortion and extramedullary haemopoiesis. Bones become enlarged, and this is most apparent in the face, with maxillary hyperplasia, dental malocclusion and development of a 'tower' skull. Extramedullary haemopoiesis can occur anywhere but is typically paraspinal and may cause compression of spinal nerves with resulting pain and weakness; intracranial haemopoiesis may cause cranial neuropathies and symptoms of raised intracranial pressure. There is an increased tendency to infection, and without transfusion the child typically dies from either infection or high-output cardiac failure. Radiography may show lacy trabecular patterns in long bones and a 'hair-on-end' appearance of the skull (Figure 14.7).

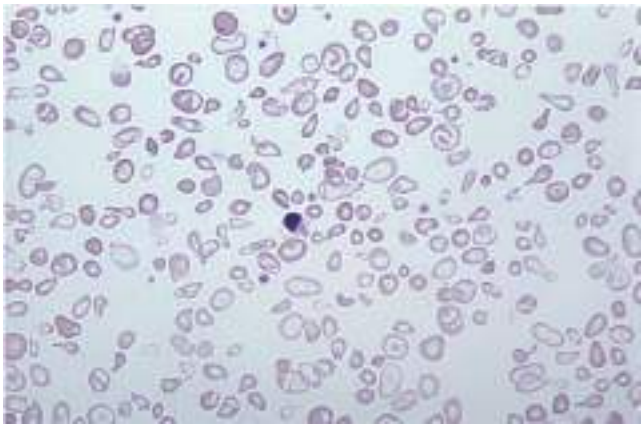

Figure 14.6 The peripheral blood appearances in β-thalassaemia.

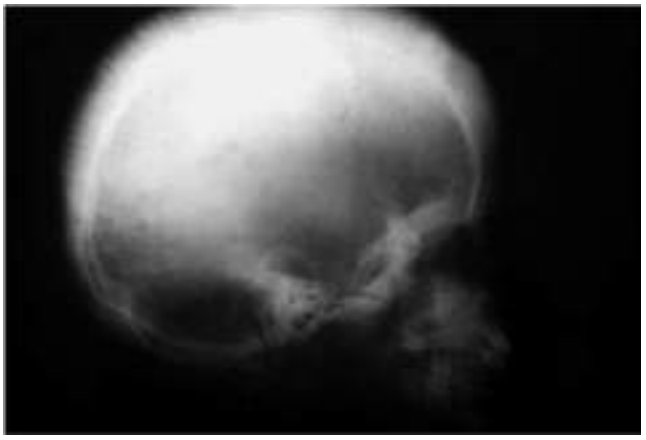

Figure 14.7 Radiograph of a skull of a thalassaemia major patient showing 'hair-on-end' appearance.

This picture is completely transformed by an appropriate transfusion regimen. The decision to start regular blood transfusions is based on clinical factors and not dependent on a particular haemoglobin level or molecular diagnosis. Occasional blood transfusions may be necessary because of an acute exacerbation of anaemia, often related to infection, and do not necessarily mean that the child is transfusion dependent. It is a bigger decision to institute a regular transfusion regimen, in that typically transfusions are then continued lifelong. Stopping transfusions in a child or adult who is adapted to a high haemoglobin level inevitably results in a prolonged period of symptomatic anaemia and ill health, which may not be tolerated. Regular transfusions should be started if the child is failing to thrive, or if erythroid expansion is causing extramedullary haemopoiesis, bony distortion or hypersplenism; abnormal facial appearances, if allowed to progress, may be irreversible without maxillofacial surgery.

Blood transfusions

The aim of regular transfusion is to correct anaemia and suppress erythroid hyperplasia. Correcting anaemia improves oxygen delivery to the tissues and facilitates normal growth and development. Suppression of erythropoiesis limits damage to bones, reduces hypermetabolism and excessive iron absorption and reduces extramedullary haemopoiesis. Measurement of soluble transferrin receptor levels, which are proportionate to the size of the erythron, suggest that suppression of erythropoiesis requires the haemoglobin to be kept above 95 g/L; in practice, this means aiming for a pretransfusion haemoglobin of 90–100 g/L. This can usually be achieved by regular red cell transfusions every 2–4 weeks, with a post-transfusion haemoglobin target of 130–150 g/L. If venous access is difficult, as is often the case in young children, it is sometimes beneficial to insert a semi-permanent central venous access device, such as a Portacath or Hickman line. The majority of thalassaemia patients are treated with simple top-up transfusions. An alternative involves regular exchange transfusion in which blood is both removed by venesection and transfused; this can be performed either manually or automatically using an apheresis machine. The main advantages are that transfusion can be less frequent, at intervals of up to 6 weeks, and iron loading is significantly less, as blood is also removed. Disadvantages include increased expense and time, increased donor exposure with risk of infection and alloimmunisation, and difficulties with venous access.

Ideally transfusions are with packed red blood cells. Leucodepleted blood reduces the risk of transfusion reactions and cytomegalovirus infection and should be used where available. When the ethnicity of the blood donor population differs from that of the recipient thalassaemia population, as occurs in most of Europe, the USA and Australia, the transfused red cells should be matched for an extended range of blood groups to minimise the risk of alloimmunisation; typically, full matching for all Rh and Kell groups, in addition to ABO, is of benefit. Blood grouping based on DNA analysis offers potential benefits, by identifying variant blood groups that may not be uncovered using serological testing. This is particularly relevant for Rh variants, which are responsible for an increasing proportion of alloantibodies as serological grouping becomes more widespread. On starting transfusions, the patient should be vaccinated against hepatitis B, and ideally also against hepatitis A.

Iron overload

Iron overload inevitably complicates regular blood transfusions and is the source of many serious complications. Each unit of transfused blood contains about 200–250 mg iron, compared with the 1 mg iron normally absorbed each day. Hepcidin is an important negative regulator of iron absorption, but in thalassaemia major and intermedia, hepcidin levels remain inappropriately low, despite increased iron stores. Erythroferrone (ERFE) is a hormone that inhibits hepcidin synthesis and is secreted by differentiating erythroblasts; greatly increased levels of ERFE were found in mice with thalassaemia intermedia, providing an explanation for the inappropriately low hepcidin and increased iron absorption. ERFE, therefore, seems to be the main cause of the low hepcidin levels encountered in β-thalassaemia where there is a large component of ineffective erythropoiesis. Growth differentiating factor-15 (GDF-15) which is produced by erythroid precursors and reaches very high levels in thalassaemia major due to erythroid hyperplasia, has previously been suggested as a pathological suppressor of hepcidin in thalassaemia, but, to date, there is no definitive evidence for its role in suppression of hepcidin (Chapters 11 and 12). Iron is initially stored in macrophages within the liver, and is chaperoned around the body bound to transferrin. As transferrin becomes saturated, labile, more toxic forms of iron appear in cells and plasma, referred to as non-transferrin-bound iron (NTBI). It is thought that NTBI is responsible for most of the iron toxicity, including iron loading into cardiac and endocrine tissues. Once in cells, the iron causes oxidative tissue damage, mostly through the generation of oxidative stress and free radicals.

Iron overload and chelation are discussed in more detail in Chapter 12. Before iron chelation was available, most regularly transfused patients with thalassaemia died in their late teens, mainly from cardiac iron deposition causing cardiac arrhythmias and heart failure. Endocrine failure was also inevitable, with diabetes mellitus, hypothyroidism, hypoparathyroidism, hypogonadism and hypopituitarism. With good iron chelation life expectancy is open-ended, and there is an established link between the efficacy of iron chelation and life expectancy.

The body has no mechanism for excreting iron and iron chelating drugs are necessary to avoid toxic iron accumulation. Iron chelation is usually started after about 1 year of monthly blood transfusions. Ideally, children delay starting chelation until they are 3 years old, as drug toxicity is thought to be highest

in the young; it is usually necessary to start chelation earlier in children who start transfusions in the first year of life, although in general low doses are used initially.

Currently, three drugs are used for iron chelation: desferrioxamine, deferiprone and deferasirox. Desferrioxamine has been in clinical use since the 1970s and is known to be safe and effective; side-effects seem only to occur when the drug is used in high doses or when iron stores are low. Important side-effects include ocular and retinal toxicity, growth impairment and cartilaginous dysplasia. The risk of *Yersinia enterocolitica* infection is increased in iron overload, particularly if desferrioxamine is also in use. Regular use of desferrioxamine has been found to prolong survival in several observational studies. The main problem is that it has to be given by injection; the most commonly used regimen involves overnight subcutaneous infusions given over 8 hours using a syringe driver or balloon-pump. To achieve negative iron balance in a regularly transfused person requires a dose of 40 mg/kg 5 times a week. The subcutaneous route is inevitably a cause of poor adherence to treatment regimens because of the pain and inconvenience. Historically, therefore, many patients have developed life-threatening iron overload despite the availability of desferrioxamine, with median age of death being 30–40 years. Deferiprone was developed in the UK about 30 years ago and was licensed in Europe in 1999, and the USA in 2011. Initial trials demonstrated its efficacy, although subsequent studies suggested that there may be individual variation in response requiring higher dosage up to 100 μg/kg/day instead of 75 μg/kg/day. Arthropathy and agranulocytosis are potentially serious side effects and it is currently recommended that people taking the drug have full blood counts every week. Despite concerns about its safety and efficacy, deferiprone seems to be effective at removing cardiac iron, which is possibly linked to improved cardiac function and reduced mortality. It is increasingly used in combination with desferrioxamine. Deferasirox is a rationally designed oral iron chelator that has been approved in the USA and Europe since the mid-2000s. Large clinical trials have demonstrated its efficacy in thalassaemia. It effectively removes hepatic iron, with increasing evidence that it also removes cardiac iron. Some studies suggest that the different iron chelators can be combined safely and that this may increase efficacy in some cases. Most frequently, deferiprone is combined with desferrioxamine to treat cardiac iron overload, although other small studies have also looked at desferrioxamine-deferasirox and deferiprone-deferasirox combinations, suggesting that they can be safely combined.

It is important to monitor iron stores regularly in transfusion-dependent patients who are receiving iron chelation. Monitoring the volume of transfused blood allows the iron input to be calculated and can be valuable when choosing the dose of iron chelator. Serum ferritin is proportional to the amount of stored iron in the liver and can be used to effectively monitor iron overload, particularly if serial measurements are used. Ferritin is artificially elevated by any inflammatory process, which can cause misleading results, particularly if there is coincidental hepatitis. Liver iron can be accurately assessed using liver biopsy, but this is invasive with a risk of complications. Increasingly, magnetic resonance imaging (MRI) is used to quantitate liver iron, with the R2 method being approved by both European and North American authorities. Cardiac MRI is an important way of identifying those patients with significant cardiac iron, which allows chelation to be targeted to the heart, using either continuous intravenous desferrioxamine or a combination of desferrioxamine and one of the oral iron chelators. Iron monitoring and chelation are very expensive and not easily available to most patients in the world with thalassaemia major.

Monitoring and annual review of patients with thalassaemia major

The aim of regular transfusions is to allow the child to grow and develop normally, and for the quality and quantity of life to be as close to normal as possible. This requires the patient to be regularly monitored, and this is ideally done in a centre with expertise in the condition, often in the form of an annual review. Each year the volume of transfused blood (expressed as mL/kg) should be recorded; if the transfusion requirement is high, exceeding 200–250 mL/kg, this suggests the possibility of hypersplenism. If the spleen is enlarged, splenectomy may significantly reduce the rate of transfusion and iron loading. Splenectomy would not normally be considered before the age of 6 years, and there is emerging concern that it may increase the risk of pulmonary hypertension and other vascular complications in later life.

Growth should be carefully monitored in children, including annual measurement of sitting height to assess spinal growth; desferrioxamine toxicity has been specifically linked to impaired spinal growth. Blood tests should be performed each year to look for endocrinopathy, including fasting glucose, thyroid stimulating hormone, parathyroid hormone, insulin-like growth factor 1 and sex hormone levels. Hepatic and renal function should be monitored regularly, together with hepatitis serology. Cardiac assessment should include an ECG, echocardiogram and increasingly MRI, which can assess both ejection fraction and iron loading; MRI is not usually tolerated by children under the age of 7 years without general anaesthesia or sedation and is not usually justified at this age unless there is strong suspicion of cardiac or other problems. Osteopenia is more common in children and adults with thalassaemia major, and bone densitometry should be monitored regularly from the age of about 10 years. Vitamin D levels should be measured regularly and optimised, with oral supplements of vitamin D being necessary in most countries. There is evidence that bisphosphonates may be effective in preventing and treating osteopenia in adults with thalassaemia, although their role in children is less established, with concern about their effects on growth. Dietary and psychological support are also often beneficial. In general, the organisation and provision of all this care require a multidisciplinary team, including paediatricians, haematologists, cardiologists, endocrinologists, nurse specialists and psychologists.

Haematopoietic stem cell transplantation in severe thalassaemia

Haematopoietic stem cell transplantation (HSCT) is usually the treatment of choice if there is an HLA-identical sibling and it is clear that the child is transfusion dependent. The success of stem cell transplantation is generally reduced as children get older, iron overload increases and iron-related organ damage increases. Under optimal circumstances, the transplant is successful in more than 90% of cases. The main complications are severe infection during the period of transplantation and either acute or chronic graft-versus-host disease. Significantly reduced fertility is also almost inevitable. One limitation of transplantation is the availability of suitable donors; cord blood transplants are potentially useful in this respect. In a small number of cases, preimplantation genetic diagnosis has allowed the selection of HLA-matched embryos to produce siblings who can act as stem cell donors. Increasingly, HSCTs are being performed using alternative sources of stem cells, particularly from haploidentical donors (parents, some siblings), or matched unrelated donors. Outcomes from these alternative donor transplants are improving but still slightly worse than from HLA-identical siblings. Nonetheless, alternative donor transplants offer an important option for some patients at the moment.

Emerging treatments

Gene therapy is starting to emerge as a viable therapeutic option for some patients. Clinical studies using a lentivirus vector to add normal copies of the beta-globin gene to stem cells from patients with severe beta thalassaemia have shown significant therapeutic benefits, increasing the haemoglobin by 20–30 g/L, and abolishing the need for transfusion in some patients who were previously transfusion dependent. This is now a commercially available treatment in some countries, although use is limited by high cost and the need for myeloablative conditioning. Gene editing approaches are still largely experimental, although a large number of different strategies are being developed. Many of these are focused on increasing gamma globin production, to compensate for the lack of beta globin, including reducing the influence of BCL11A on gamma globin transcription and a commercial treatment based on this approach has recently been licensed in Europe, the USA and some other regions. A very small number of patients have so far been treated in this way, with encouraging results. Progress in this area continues to be very rapid, with more precise ways of gene editing being developed, novel targets being identified and new ways of delivering the vectors to erythropoietic tissue to avoid the need for myeloablative conditioning. In the future, it seems likely that stem cell treatment in thalassaemia will involve the in vivo delivery of gene editing tools that can specifically correct the beta-globin variant causing thalassaemia.

Luspatercept is a recombinant protein designed to bind to transforming growth factor-beta ligands to improve red cell maturation, via the inhibition of SMAD2 and SMAD3 signalling. A randomised, placebo-controlled clinical trial in patients with transfusion-dependent beta thalassaemia found that about 20% of patients had a reduction in their transfusion requirements of at least 33% during the study, and 11% of patients became transfusion-independent. There are likely to be similar benefits for patients with thalassaemia intermedia. Luspatercept is approved for use in the EU and USA, although it is not curative and it is not yet clear exactly how it should be used in thalassaemia. It potentially reduces the transfusion requirements of patients, and may reduce iron accumulation, but this has to be balanced against the cost of the drug and its potential side-effects, which include an increased risk of thromboembolism and increased extramedullary haematopoiesis. On-going clinical trials also suggest that pyruvate kinase activators may reduce transfusion requirements in some patients with transfusion dependent thalassaemia.

Prognosis in severe thalassaemia

In theory, with adequate transfusion and effective chelation, survival in thalassaemia major should approach that of the normal population, although historically this has never been the case because of the difficulties patients encounter in adhering to iron chelation regimens, particularly with subcutaneous infusions. In Europe and North America, median survival has improved with each decade since the introduction of desferrioxamine in the 1970s. Most deaths are related to cardiac iron overload and improved survival is primarily related to earlier and more rational use of chelation. Recent studies suggest that deaths from cardiac complications have become much less common in the last 15 years, probably related to improved awareness and treatment of iron-related cardiomyopathy with intravenous desferrioxamine and combination therapy with both desferrioxamine and deferiprone. Earlier detection, using cardiac T2* MRI, and prevention of cardiac iron loading, with oral chelation, seem likely to further improve prognosis. Liver disease, malignancy and infection are becoming relatively more common causes of death.

Heterozygous β-thalassaemia

Carriers for β-thalassaemia are usually symptom-free except in periods of stress such as pregnancy, when they may develop significant anaemia. Transfusion is sometimes necessary during pregnancy, but no other treatment or follow-up is necessary. Palpable splenomegaly is rare. Carriers may have mild anaemia; haemoglobin values are in the range 90–120 g/L. The red cells show hypochromia and microcytosis proportionately greater than the degree of hypochromia seen in iron deficiency. The reticulocyte count is normal. The bone marrow shows mild erythroid hyperplasia with corresponding slight increase in soluble transferrin receptor levels. The characteristic finding is an elevated HbA_2 level, usually greater than 3.5%. There is a slight elevation of HbF in the 1–3% range in about 50% of cases. Silent

or near-silent carriers of β-thalassaemia occur fairly rarely, in which the HbA_2 level is normal with only slight hypochromia; this is typically associated with mild thalassaemic variants in the promoter region of the gene. Failure to identify this state antenatally can result in significant and unexpected thalassaemia in the offspring, if the partner also carries β-thalassaemia. Borderline increases of HbA_2 with variable increases of HbF, but normal red cell indices have also been associated with variants in the *KLF1* gene, but the clinical significance of such variants when coinherited with β-thalassaemia is not clear.

β-Thalassaemia in association with haemoglobin variants

In many populations, because there is a high incidence of both β-thalassaemia and other globin gene mutations, it is quite common for an individual to inherit a β-thalassaemia allele from one parent and a gene for a structural haemoglobin variant from the other. Although numerous interactions of this type have been described, only three are common and significant: HbS/β-thalassaemia, HbC/β-thalassaemia and HbE/β-thalassaemia.

HbS/β-thalassaemia

HbS/β-thalassaemia causes sickle cell disease, with the severity varying from very mild to severe (see also Chapter 15). This interaction does not result in clinically significant thalassaemia. The principal determinant of the severity of sickle cell disease is the nature of the β-thalassaemia mutation, and to what extent β^A is reduced compared with normal. HbS/β^0 -thalassaemia with no HbA present and an average haemoglobin of 70–80 g/L is broadly similar in severity to HbSS. It differs in that red cells are markedly hypochromic and contain excess α-globin chains. Clinically significant splenomegaly is more common than in HbSS, affecting up to 30% of adults. The severity of HbS/β^+-thalassaemia varies from severe to very mild, depending on how much HbA is produced. Some β^+-thalassaemia alleles produce less than 10% of the normal HbA output and result in a clinical picture identical to HbS/β^0-thalassaemia. At the other end of the spectrum, promoter mutations typically reduce HbA output by only 10% and result in a condition that is only marginally more symptomatic than sickle cell carriers. These mild forms of HbS/β^+-thalassaemia occur most often in populations of African origin, whereas the more severe forms are most often found in Mediterranean countries and India.

HbC/β-thalassaemia

HbC is common in parts of West Africa and occurs to a minor extent in populations of North African and southern Mediterranean origins. It is largely asymptomatic and characterized by a mild haemolytic anaemia associated with splenomegaly. The peripheral blood film shows numerous target cells and thalassaemic red cell changes with a moderately elevated reticulocyte count. Haemoglobin electrophoresis shows a preponderance of HbC. The diagnosis is confirmed by finding the HbC trait in one parent and the β-thalassaemia trait in the other, and by DNA analysis where appropriate.

HbE/β-thalassaemia

This is the commonest severe form of thalassaemia in Southeast Asia and parts of the Indian subcontinent. The β^E allele is mildly thalassaemic due to the activation of a cryptic splice site, and when it is inherited together with β^0-thalassaemia, there is a marked deficiency of β-chain production. HbE is slightly unstable *in vitro*, although it is not clear how clinically significant this is. The clinical and haematological features are very variable. There are thalassaemic red cell changes, and the bone marrow shows marked erythroid hyperplasia. There is nearly always anaemia and splenomegaly, with typical thalassaemic bone changes. Haemoglobin values are in the range 40–100 g/L, with an average of 60–70 g/L; in well-resourced countries about half of the patients with this condition are regularly transfused. There are thalassaemic red cell changes and the bone marrow shows marked erythroid hyperplasia.

Although relatively little is known about the natural history of this disorder, it is clear that in many parts of Southeast Asia and India, it causes a very high mortality in early life. Clinically it is indistinguishable from other forms of β-thalassaemia, although the link between genotype and phenotype is less predictable, with some patients having thalassaemia major and others, being non-transfusion dependent, growing and developing with few complications.

The diagnosis is confirmed by finding only HbE and HbF on haemoglobin electrophoresis and by demonstrating HbE trait in one parent and β-thalassaemia trait in the other. In cases of HbE/β^+-thalassaemia, variable quantities of HbA are present and the condition is usually milder. Neonatal diagnosis can be difficult, particularly distinguishing between homozygotes for HbE and HbE/β^0-thalassaemia; family studies and DNA analysis are useful in these circumstances.

Variant forms of β-thalassaemia

Despite the vast heterogeneity of mutations, the increased levels of HbA_2 in β-thalassaemia heterozygotes are remarkably uniform, in the range 3.5–5.5%, and rarely exceeds 6%. Unusually high HbA_2 levels in excess of 6.5% seem to characterise the subgroup caused by lesions (point mutations or small deletions) that affect the regulatory elements in the promoter region of the β-globin gene (see Figure 14.4). The unusually high HbA_2 levels are usually accompanied by higher than usual increases in HbF, resulting in a milder thalassaemia phenotype, despite the absence of HbA in some cases. Otherwise, the haematological picture is identical to the common forms of β thalassaemia.

Some β-thalassaemia heterozygotes have normal HbA_2 levels, despite the typical hypochromic microcytosis. In most cases, this is due to the coinheritance of δ-thalassaemia. Other cases of normal HbA_2 β-thalassaemia are extremely mild forms of β-thalassaemia that is completely silent in heterozygotes and is only identified when it is coinherited with a common form of β-thalassaemia. Heterozygotes do not have any evident haematological phenotype, the only abnormality being a mild imbalance of globin chain synthesis.

β-Thalassaemia occasionally follows a dominant pattern of inheritance, producing symptoms in heterozygotes (see Figure 14.3). The clinical picture is characterised by a moderate degree of anaemia and splenomegaly with marked thalassaemic changes of the red cells, and ineffective erythropoiesis with intracellular inclusion bodies. Although not usually transfusion-dependent, such individuals develop iron overload from hyperabsorption due to ineffective erythropoiesis and may develop liver and/or endocrine damage. The common denominator of these dominantly inherited β-thalassaemias is the synthesis of hyper-unstable β-chain variants, caused by a spectrum of mutations; those resulting from PTCs are typically found in exon 3 of the β-globin gene. Unlike the recessive forms that are prevalent in malarious regions, dominantly inherited β-thalassaemias are rare, occurring in dispersed geographical regions. Most of the dominant β-thalassaemia alleles have been described in single families, many as *de novo* mutations. Dominantly inherited β-thalassaemia should be suspected in any patient with a thalassaemia intermedia phenotype, even if both parents are haematologically normal, and the patient is from an ethnic background where β-thalassaemia is rare. The diagnosis is confirmed by DNA sequence analysis of the β-globin genes.

δβ-Thalassaemia and hereditary persistence of fetal haemoglobin

HPFH and δβ-thalassaemia are much less common than β-thalassaemia and manifest as a range of disorders characterised by decreased or absent HbA production and a variable compensatory increase in HbF synthesis. The distinction between them is subtle and originally made on what appeared to be clear-cut clinical and haematological grounds. However, as more cases became recognised and their underlying mutations delineated, it became evident that there is considerable overlap between the two groups of disorders.

The level of compensatory increase is higher in HPFH compared with δβ-thalassaemia. HPFH heterozygotes have essentially normal red cell indices, normal HbA_2 levels and HbF levels of 10–35%, whereas heterozygotes for δβ-thalassaemia have hypochromic microcytic erythrocytes and normal HbA_2 levels and the HbF increases are lower (5–15%). A distinguishing feature is the heterocellular distribution of HbF in δβ-thalassaemia compared with a pancellular (or homogeneous) distribution in HPFH, although the intercellular distribution of HbF may be a reflection of the magnitude of increase and the sensitivity of the technique used to stain the HbF in the red blood cells (F cells). Clinically, HPFH homozygotes are asymptomatic with slightly reduced or normal MCV and MCH, and compound heterozygotes with β-thalassaemia have very mild disease. Compound heterozygotes of δβ-thalassaemia with β-thalassaemia, and δβ-thalassaemia homozygotes have disease severity that ranges from mild anaemia to transfusion dependence.

Two types of mutations underlie this group of disorders: deletions that remove substantial regions of the β-globin cluster, including the β-globin gene (Figure 14.8), and point mutations in the promoters of either of the γ-globin genes ($^{G}\gamma$ or $^{A}\gamma$). Six deletion forms of HPFH have been described in Africans, Mediterraneans, Indians and Southeast Asians, with deletions ranging from 13 to 86 kb in size. The commonest forms are Black HPFH-1 and HPFH-2, the latter also referred to as Ghanaian HPFH. Included within the HPFH conditions is Hb Kenya, a rare condition found largely in East Africa and characterised by production of a hybrid $^{A}\gamma\beta$-globin chain and increased $^{G}\gamma$-chain (Figures 14.8 and 14.9). The first type of δβ-thalassaemia to be described was Hb Lepore, which consists of hybrid δβ-globin chains produced by misaligned crossing-over between the δ- and β-globin genes (Figures 14.8 and 14.9). Heterozygotes for Hb Lepore have hypochromic microcytic red cells, normal HbA_2 and variably increased HbF. The more common forms of δβ-thalassaemia result from deletions of different lengths in the β-globin gene cluster (Figure 14.8). Deletions that leave both the γ-globin genes intact are called $^{G}\gamma^{A}\gamma$ $(\delta\beta)^0$ -thalassaemias, and they all include parts or all of the δ- and β-globin genes. $^{G}\gamma(^{A}\gamma\delta\beta)^0$ -thalassaemia deletions include part or all of the $^{A}\gamma$-globin gene and hence produce HbF containing only $^{G}\gamma$-chains. A couple of the $(\delta\beta)^0$-thalassaemias include two deletions separated by an inverted region.

Non-deletion HPFH is caused by mutations within the promoters of either of the γ-globin genes and lead to variably increased HbF with a preponderance of only one of the γ-chains. The mutations (single-base substitutions or minor deletions) are clustered in three regions of the promoters, around positions −114, −175 and −200. These regions contain binding sites for ubiquitous and erythroid-specific factors. Altered binding patterns of the transcription factors due to the point mutations are thought to be the cause of the elevated HbF levels, which vary from 5 to 35% in heterozygotes. Deletion and non-deletion HPFH and δβ-thalassaemias are clearly inherited in a Mendelian fashion as alleles of the β-globin gene complex. Heterozygotes have significant elevations of HbF ranging from 10 to 40%.

Inherited increases in HbF have also been reported in families with β-thalassaemia and sickle cell disease; these increases had a modulating effect on the severity of disease and the inheritance appeared to segregate independently of the β-globin gene cluster. Healthy members of the families may also have slight increases in HbF, and historically these individuals were said to have coinherited heterocellular HPFH. Heterocellular HPFH

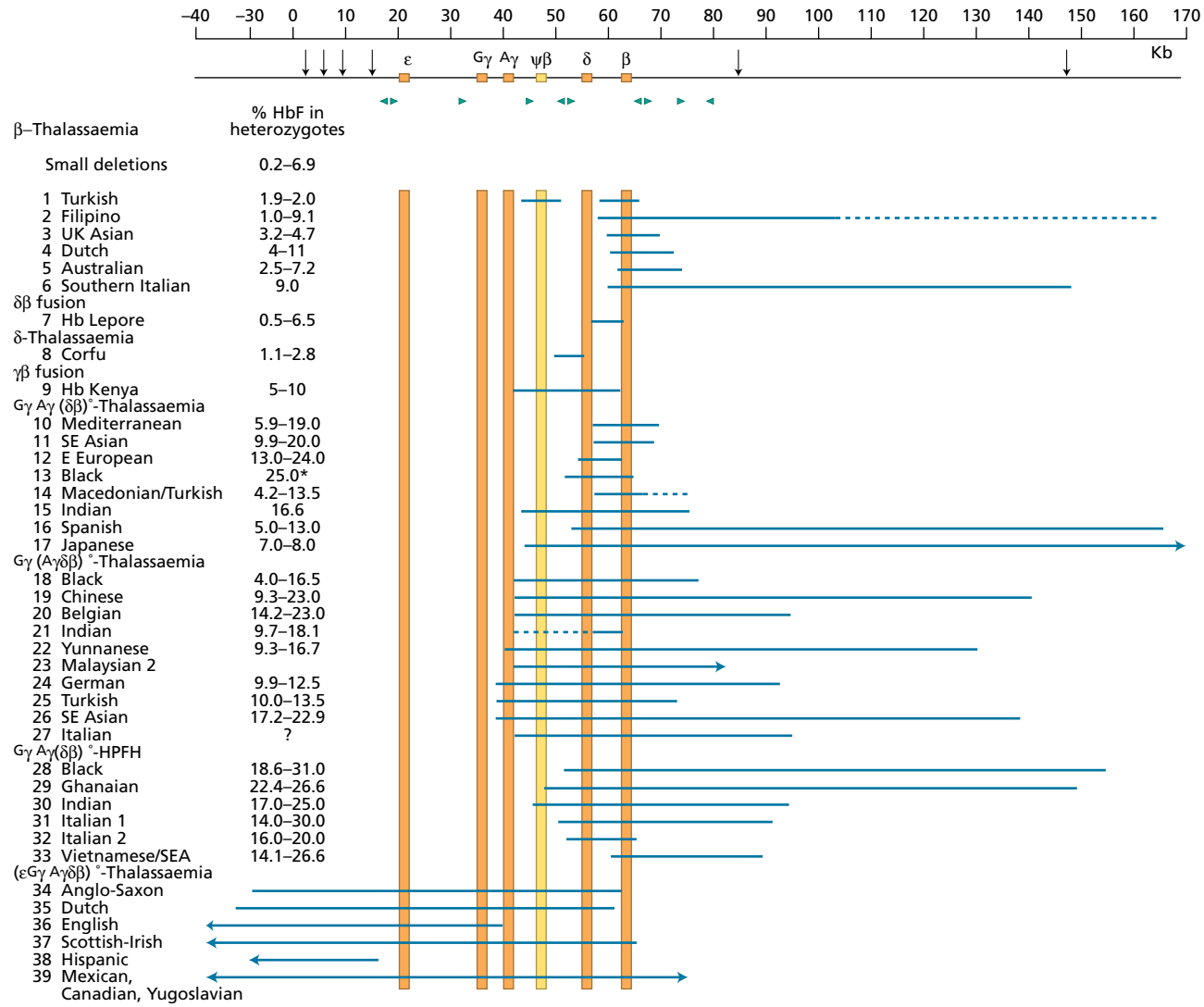

Figure 14.8 The deletions that underlie δβ-thalassaemia and hereditary persistence of fetal haemoglobin. The upper arrows represent DNase I-hypersensitive sites (Source: Weatherall and Clegg 2001. Reproduced with permission of WHO.)

was originally recognised in a group of Swiss army recruits; the HbF elevation was modest and unevenly distributed amongst the erythrocytes, the distinct HbF-carrying red blood cells being termed F cells. Hence Swiss HPFH was also referred to descriptively as heterocellular HPFH. It is now clear that HbF is a highly variable quantitative trait, and that heterocellular HPFH represents the upper tail of the natural continuous distribution that includes approximately 10% of the population with HbF levels between 0.8 and 5%. Unlike the Mendelian forms caused by major deletions or point mutations in the γ-globin promoters, the inheritance of heterocellular HPFH is complex, with an overwhelming genetic contribution. The sequence variant (C→T) at position −158 of the ^{G}γ-globin gene, also referred to as the *Xmn*1-^{G}γ site (or rs74821440), was the first QTL to be implicated through family studies but subsequent studies indicate that rs74821440 is likely to be a surrogate marker for yet to be identified variant(s) in the HBB cluster. Subsequent genetic association studies have confirmed *Xmn*1-^{G}γ as one of the three major QTLs modulating HbF production in adults, the other two being the *HBS1L-MYB* intergenic region on chromosome 6q and *BCL11A* on chromosome 2p. These three QTLs account for a relatively large proportion (20–50%) of the common variation in HbF levels, not only in healthy adults, but also in patients from diverse ethnic groups – Brazilians, African–Americans, African–British, Tanzanians with sickle cell disease. In African–American patients with sickle cell disease, the three loci contribute more than 20% to the HbF variation with a corresponding reduction in frequency of acute pain. Coinheritance of the *Xmn*1-^{G}γ site delays transfusion requirements in β-thalassaemia. A combination of the three HbF QTLs and α-globin genotype

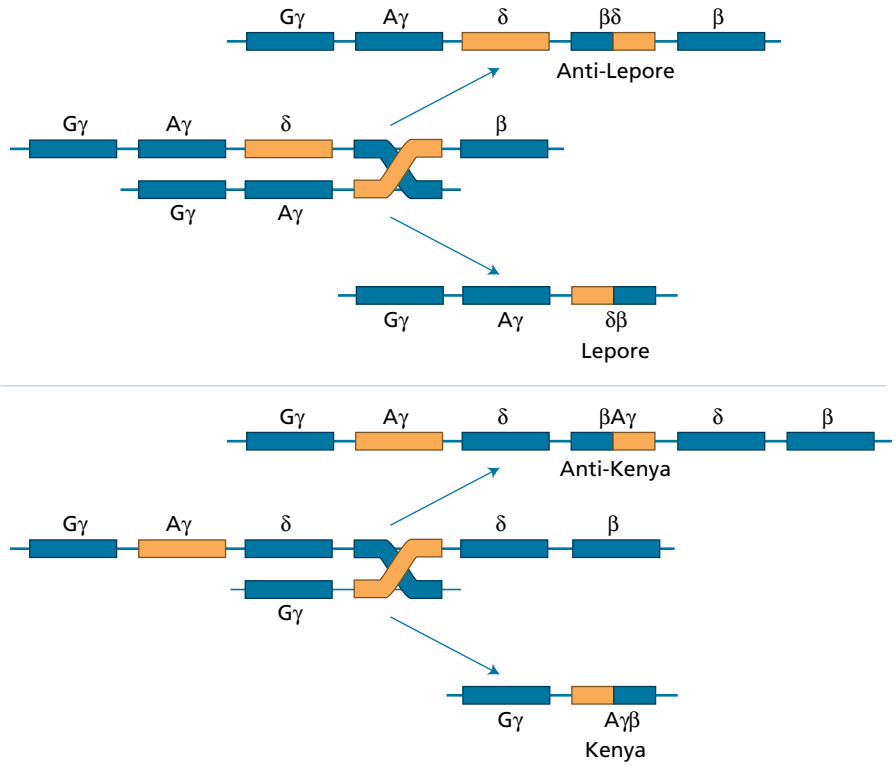

Figure 14.9 The mechanisms for the production of haemoglobin Lepore and related variants.

have also been shown to be predictors of disease severity and transfusion requirements in patients with β-thalassaemia and HbE/β-thalassaemia. HbF increases secondary to *KLF1* variants, either as a primary phenotype or in association with other red cell disorders, such as congenital dyserythropoietic anaemia (CDA type IV), are increasingly noted.

εγδβ-Thalassaemia

These are rare conditions and result from large deletions of the β-globin gene cluster that involve the β-LCR. The deletions fall into two main categories: group I removes all, or a greater part of, the complex including the β-globin gene and the β-LCR, and group II removes extensive upstream regions including the β-LCR but leaving the β-globin gene itself intact. There is no output from the globin genes of the affected cluster. Clearly, the homozygous state would not be compatible with survival. Heterozygotes have severe haemolytic disease of the newborn, with anaemia and hyperbilirubinaemia. The severity of anaemia and haemolysis is variable, even within a family, and in some cases, blood transfusions are necessary during the neonatal period. If they survive the neonatal period, the infants grow and develop normally as compared with infants with typical β-thalassaemia trait. Affected adults have the haematological picture of heterozygous β-thalassaemia, with mild anaemia, hypochromic microcytic red cells and a haemoglobin pattern of normal HbA_2 β-thalassaemia. Using next-generation sequencing (NGS) technology, a novel form of εγδβ-thalassaemia due to an inversion-deletion has recently been characterised in three unrelated English families.

The α-Thalassaemias

Distribution

The α-thalassaemias follow a similar distribution to the β-thalassaemias, extending throughout sub-Saharan Africa, the Mediterranean region, the Middle East, the Indian subcontinent and Southeast Asia, in a line stretching from southern China through Thailand, the Malay Peninsula and Indonesia, to the Pacific Island populations.

In some prevalent areas, carrier frequency for the mild form (α^+) reaches 80% or more. The more severe forms (α^0-thalassaemias) reach their highest frequency in Southeast Asia, where carrier frequency can reach 10%.

Genetic basis of disease: molecular pathology

Normal individuals have four α-globin genes arranged as linked pairs, α2 and α1, at the tip of each chromosome 16, the normal α-genotype being represented as αα/αα (Figure 14.10). The α-thalassaemias can be classified as α^0-thalassaemia, in which no α-chains are produced from the linked pair, and α^+-thalassaemia, in which production of α-chain from the affected chromosome is reduced.

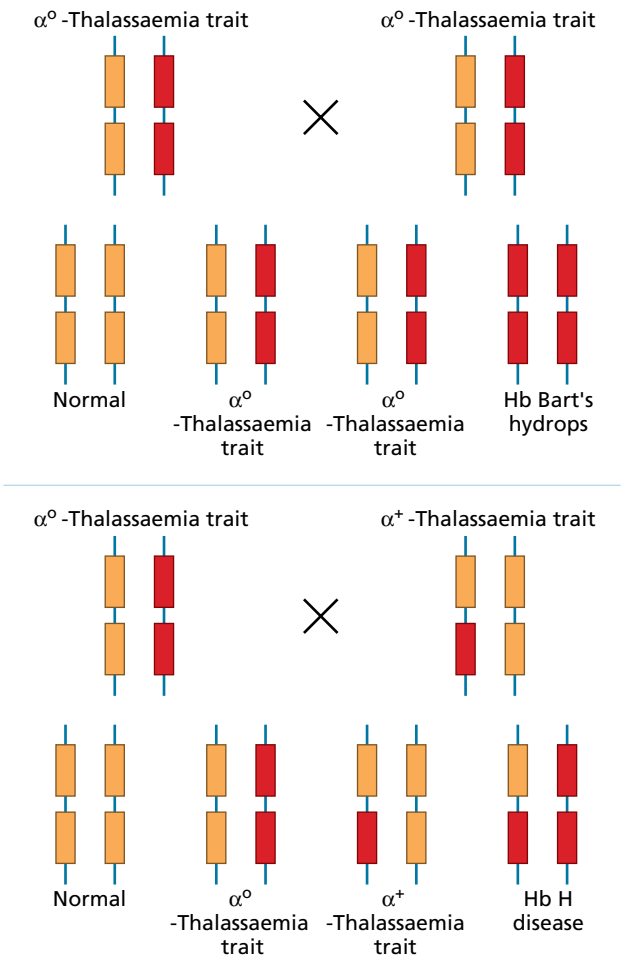

Figure 14.10 The genetics of α-thalassaemia.

The α^0-thalassaemias are caused by deletion of both α-globin genes (Figure 14.11). The deletions vary in size and tend to be geographically isolated, with two particularly common ones in Southeast Asia (/– $-^{SEA}$) and the Mediterranean region (/– $-^{MED}$). Rarely, α^0-thalassaemia can also arise from deletions of the upstream α-globin regulatory elements (MCS) in which the α-globin genes remain intact but completely inactivated. In other cases, more extensive deletions are associated with monosomy for a segment of the tip of chromosome 16p that, when greater than about 1 megabase (Mb), result in the alpha thalassaemia X-linked intellectual disability syndrome (ATR-16). The molecular basis of the α^+-thalassaemias is more complicated; the commonest forms result from deletions that remove one of the linked pairs of α-globin genes, leaving the other intact (–α/αα). The linked α-globin genes are embedded in two highly homologous duplicated units of 4kb within which are three homologous subsegments designated X, Y and Z (Figure 14.12), misalignment and recombination between the Z segments, which are 3.7kb apart, produces chromosomes with one α-globin gene (/$-\alpha^{3.7}$ or *rightward* deletion) and others with three α-globin genes (/ααα$^{anti\text{-}3.7}$). Similarly, crossover between the X boxes, which are 4.2kb apart, produces the *leftward* deletion /$-\alpha^{4.2}$ and the /ααα$^{anti\text{-}4.2}$ allele.

Less commonly, both the α-globin genes are intact and α^+-thalassaemia results from point mutations that partially or completely inactivate one of them ($\alpha^T\alpha/\alpha\alpha$). In contrast to the β-thalassaemias, non-deletional mutations are much less common causes of α-thalassaemia but, like those that cause β-thalassaemia, the non-deletional α-thalassaemia variants act at different stages of gene regulation and expression. The majority of the non-deletional α-thalassaemia (more than two-thirds) are found on the dominant α2 gene, less than one-third on the α1 gene and the others on an /–α-chromosome (/$-\alpha^T$). In general, the non-deletional α^+-thalassaemia variants (/$\alpha^T\alpha$) give rise to a more severe reduction in α-chain output than the single α-gene deletion (/–α) due to the lack of compensatory increase in α-output from the linked α1 gene, as observed in deleted cases. One particularly common form of non-deletional α-thalassaemia found in Southeast Asia is Hb Constant Spring (/$\alpha^{CS}\alpha$), which is due to a single-base substitution (TAA→CAA) in the α_2-globin termination codon. Instead of terminating at the stop codon, mRNA is read through the 3′-UTR until another in-phase termination codon is encountered 31 codons later. This results in an elongated α-globin chain of 172 residues, 31 amino acids from the natural arginine at codon 141. Of the six predicted α_2-chain termination variants, five have been described: Hb Constant Spring (α142 Gln), Hb Icaria (α142 Lys), Hb Koya Dora (α142 Ser), Hb Seal Rock (α142 Glu) and Hb Pakse (α142 Tyr). Hb Constant Spring is by far the most common of these variants, reaching frequencies of up to 4% in Thailand. Finally, non-deletional α-thalassaemia can also arise from single-base substitutions causing structural α-globin variants that are highly unstable, for example Hb Quong Sze α125 Leu→Pro (/α^{QS} α).

Unusual causes of α-thalassaemia

This includes a single case report of α^0-thalassaemia arising from a deletion involving the α1 gene that also inactivated the intact linked α2 gene. Subsequent studies showed that the deletion results in juxtaposition of a downstream gene (*LUC7L*) next to the structurally normal upstream α2 gene. Transcription of antisense mRNA from *LUC7L* led to silencing of the linked α2 gene. Another novel form of non-deletional α-thalassaemia results from a single nucleotide substitution in a non-genic region between the α-globin genes and their upstream regulatory elements. The single-base substitution leads to the creation of a new promoter-like element that interferes with normal activation of all the downstream α-like globin genes, resulting in α^0-thalassaemia.

Pathophysiology

The pathophysiology of α-thalassaemia is different from that of β-thalassaemia. A deficiency of α-chains leads to the production of excess γ- or β-chains, which form Hb Bart's (γ_4) and HbH (β_4),

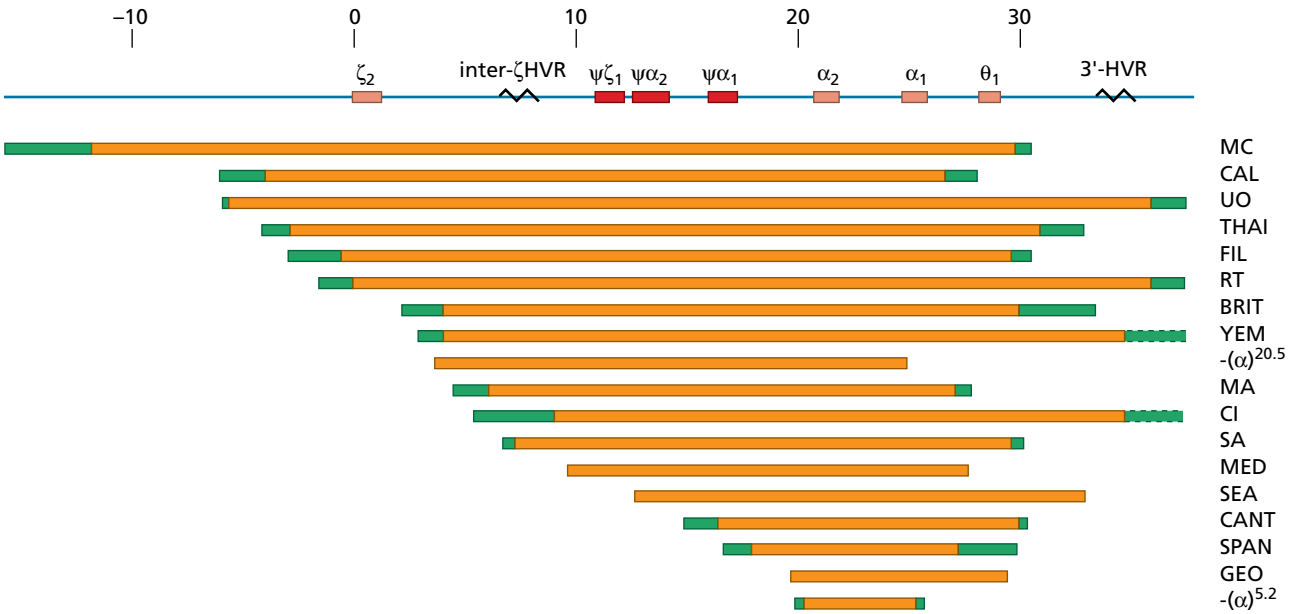

Figure 14.11 The α-globin gene cluster deletions that underlie α^0 thalassaemia (Source: Weatherall and Clegg 2001. Reproduced with permission of WHO.)

respectively. These soluble tetramers do not precipitate extensively in the bone marrow and hence erythropoiesis is more effective than in β-thalassaemia. However, HbH is unstable and precipitates in red cells as they age. The inclusion bodies cause red cell membrane damage and obstruction in the spleen leading to shortened red cell survival. Furthermore, both Hb Bart's and HbH have a very high oxygen affinity and their oxygen dissociation curves resemble myoglobin. Thus, the severe forms of α-thalassaemia are due to defective haemoglobin production, the synthesis of homotetramers that are physiologically useless, and a haemolytic component.

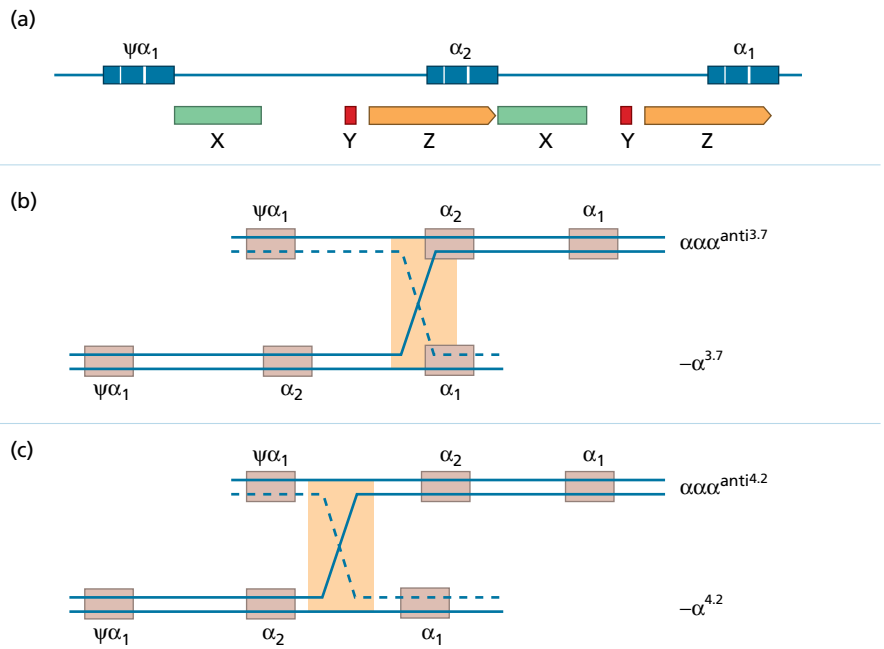

Figure 14.12 The molecular mechanisms that underlie the deletion forms of α-thalassaemia: (a) normal cluster showing X, Y and Z homology boxes. (b) 3.7-kb deletion. (c) 4.2-kb deletion (Source: Weatherall and Clegg 2001. Reproduced with permission of WHO.)

Genotype–phenotype relationship

Loss of one functioning α-gene (αα/−α) is almost completely silent, with normal or only slightly hypochromic red cells. Loss of two α-genes (− −/αα or −α/−α) produces a mild hypochromic microcytic anaemia, the α-thalassaemia trait. Homozygotes for α^0-thalassaemia (− −/− −) have a lethal condition with intrauterine haemolytic anaemia called Hb Bart's hydrops fetalis syndrome (see Figure 14.10). As Hb Bart's hydrops fetalis syndrome follows the homozygous inheritance of α^0 thalassaemia, this condition occurs only in populations in which α^0-thalassaemia is common, notably those of Southeast Asia and the Mediterranean islands. Deficiency of α-chains gives rise to an excess of γ-chains (in fetal life) or β-chains (in adult life), which form γ_4 tetramers (Hb Bart's) or β_4 tetramers (HbH). The presence of Hb Bart's or HbH is thus diagnostic of α-thalassaemia. Due to their very high oxygen affinity, Hb Bart's and HbH are not functional haemoglobins; HbH is unstable and precipitates in older red cells, forming H inclusions.

HbH disease lies between the two ends of the clinical spectrum, the asymptomatic α-thalassaemia trait and Hb Bart's hydrops fetalis As in β-thalassaemia intermedia, HbH disease spans a wide range of clinical and haematological phenotypes, with equally heterogeneous genotypes, varying with the geographic distribution of the different α-thalassaemia variants. HbH disease most commonly results from the interaction of α^0 and α^+-thalassaemia (− −/−α) and thus, similar to Hb Bart's hydrops syndrome, is also restricted to populations where α^0 -thalassaemia is common. Less often, it can result from the interaction of α^0 -thalassaemia with non-deletional forms of α-thalassaemia (/α^Tα) or from homozygous non-deletional α-thalassaemia ($\alpha^T\alpha/\alpha^T\alpha$). HbH disease in Southeast Asia commonly arises from homozygosity or compound heterozygosity for Hb Constant Spring ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$ or $\alpha^{CS}\alpha$/− −).

As the non-deletional forms of α^+-thalassaemia tend to have a more severe phenotype than the deletional forms, in some cases the homozygous state ($\alpha^T\alpha/\alpha^T\alpha$) may be associated with the phenotype of HbH disease. In a small number of cases, HbH can be unusually severe, requiring regular blood transfusion, and rare cases may result in Hb Bart's hydrops syndrome (in particular genotype − −/α^T α).

Haemoglobin Bart's hydrops syndrome

This is an important cause of fetal loss throughout Southeast Asia and the eastern Mediterranean. There is no production of α-chains and hence neither fetal nor adult haemoglobin. The fetus is usually stillborn between 28 and 40 weeks or, if liveborn, takes a few gasping respirations and then expires within the first hour after birth. Affected neonates show the typical picture of hydrops fetalis, with gross pallor, generalised oedema and massive hepatosplenomegaly. Fetal anaemia may be detected from early in the second trimester, with increased blood flow on the fetal middle cerebral artery, as measured by Doppler ultrasound. There is an increased frequency of congenital abnormalities and a very large friable placenta. All these findings are due to severe intrauterine anaemia. The haemoglobin is in the range of 60–80 g/L and there are gross thalassaemic changes in the red cells, with many nucleated erythroid cells in the blood. The haemoglobin consists of approximately 80% Hb Bart's and 20% Hb Portland ($\zeta_2\gamma_2$). Very rarely, Hb Bart's hydrops infants have survived to term because they continue to produce embryonic haemoglobin. Apart from fetal death, this syndrome is characterised by a high incidence of toxaemia of pregnancy and obstetric complications due to the large placenta.

Haemoglobin H disease

This condition is characterised by a variable degree of anaemia and splenomegaly, but it is unusual to find severe thalassaemic bone changes or growth retardation. Patients usually survive into adult life, although the course may be interspersed with severe episodes of haemolysis associated with infection or worsening of the anaemia due to progressive hypersplenism. Parvovirus (erythrovirus) B19 infection, in particular, may cause transient reticulocytopenia with severe anaemia and the need for red cell transfusion. In addition, oxidant drugs may increase the rate of precipitation of HbH and exacerbate the anaemia. Haemoglobin values range from 70 to 100 g/L and the blood film shows typical thalassaemic changes. There is a moderate reticulocytosis and, on incubation of the red cells with brilliant cresyl blue, numerous inclusion bodies are generated by precipitation of HbH under the redox action of the dye. Extramedullary haematopoiesis can be a problem, particularly in more severe forms associated with non-deletional variants. After splenectomy, large preformed inclusions can be demonstrated on incubation of blood with methyl violet. Haemoglobin analysis reveals 5–40% HbH, together with HbA and a normal or reduced level of HbA_2.

α-Thalassaemia traits

α^0-Thalassaemia trait is characterised by very mild hypochromic anaemia with red cell indices similar to those of β-thalassaemia trait; the MCH is less than 25 pg and the HbA_2 level is normal. Occasional HbH bodies may be present in the red cell on supravital staining. There are no diagnostic tests with which to identify this condition with certainty except DNA analysis. Deletional α^+-thalassaemia carriers have near-normal haematological findings. The heterozygous states for the non-deletional forms of α^+ -thalassaemia are sometimes associated with very mild hypochromic anaemia; the type associated with Hb Constant Spring can be identified by the presence of trace amounts of the variant on haemoglobin electrophoresis at an alkaline pH. α^0-Thalassaemia carriers can be identified with more certainty in the neonatal period when they have 5–10% Hb Bart's, which disappears over the first few months of life and is not replaced by HbH. Some α^+-thalassaemia

carriers have slightly increased levels of Hb Bart's, in the 1–3% range, but its absence does not exclude the diagnosis.

Other forms of α-thalassaemia

There are several other forms of α-thalassaemia that are completely unrelated in their pathogenesis and distribution to the conditions described in the previous sections. They comprise the α-thalassaemia with intellectual disability syndromes and the association of α-thalassaemia with myelodysplasia.

α-Thalassaemia with X-linked intellectual disability syndromes

There are two forms of α-thalassaemia associated with intellectual disability, one arising from mutations on chromosome 16 (ATR-16) the other, on the X chromosome (ATR-X). ATR-16 results from large chromosomal rearrangements and extensive deletion of 1–2 Mb from the subtelomeric end of the short arm of chromosome 16. Affected children usually have a relatively mild degree of intellectual disability and no dysmorphic features. Because the *ATR-X* gene is on the X-chromosome and X-linked, it affects boys, and is associated with widespread dysmorphic features and severe intellectual disability. The condition results from many different mutations of the *ATRX* gene. The ATRX protein has many features in common with DNA helicases, transcription factors that are involved in the modelling of chromatin and gene regulation. ATRX also appears to play an important role in the transcription of the α-globin genes and undoubtedly many other genes during early development. The *ATRX* gene has also been reported to be involved in a considerable number of X-linked intellectual disability syndromes without an α-thalassaemia phenotype.

α-Thalassaemia associated with myelodysplasia

An α-thalassaemic phenotype is also found in association with forms of myelodysplasia in elderly patients. The blood films of such patients show dimorphic features, with populations of red cells containing HbH inclusion bodies and a variable level of HbH in peripheral blood. Acquired mutations in the *ATRX* gene have been identified in the blood cells of patients with this syndrome. The relationship of the mutations in *ATRX* to the neoplastic phenotype remains to be determined.

Thalassaemia intermedia (non-transfusion-dependent thalassaemia)

Definition and molecular pathology

The term thalassaemia intermedia is used to describe patients with the clinical picture of thalassaemia which, although not transfusion dependent, is associated with a more severe degree of anaemia than is found in carriers for α- or β-thalassaemia. It is increasingly referred to as non-transfusion-dependent thalassaemia (NTDT), particularly in literature discussing iron chelation. Whether a patient is classified as having thalassaemia intermedia or thalassaemia major depends on a doctor deciding that the patient would benefit from regular blood transfusions; this decision is based not only on the clinical evidence mentioned in the section on thalassaemia major, but also on more complex factors such as the availability of safe blood transfusions, the experience of the clinician and the wishes of the patient. Many different genotypes underlie thalassaemia intermedia, as mentioned earlier, with HbE/β-thalassaemia perhaps being the commonest (Table 14.2). HbH disease is sometimes considered as a type of thalassaemia intermedia, but its pathophysiology is quite different to that caused by β-thalassaemia.

Table 14.2 Molecular basis of β-thalassaemias intermedia.

Homozygous or compound heterozygous state for β-thalassaemia
Inheritance of mild β-thalassaemia alleles (homozygous or compound heterozygotes)
Compound heterozygosity for a mild and a more severe allele
Coinheritance of α-thalassaemia
Increased HbF response
β-Globin gene promoter mutations (deletional or non-deletional)
Coinheritance of HbF quantitative trait loci
Linked: Xmn1- $^{G}\gamma$ polymorphism
Unlinked: *BCL11A* gene (chromosome 2p), *HBS1L-MYB* intergenic polymorphisms (chromosome 6q) *KLF1,* variants
Heterozygous state for β-thalassaemia
Coinheritance of extra α-globin genes as triplicated (/ααα) or quadruplicated (/αααα) globin complexes or segmental duplication of entire α-globin cluster
Dominantly inherited β-thalassaemia (hyper-unstable β-globin chain variants)
Compound heterozygosity for β-thalassaemia and β-chain variants
HbE/β-thalassaemia
Compound heterozygosity for β-thalassaemia and HPFH or δβ-thalassaemia
Homozygosity for δβ-thalassaemia

Clinical and haematological changes

At one end of the spectrum are individuals who, except for mild anaemia, are symptom-free. At the other, there are patients who have haemoglobin values of 50–70 g/L and who develop marked splenomegaly, osteopenia and skeletal deformities due to expansion of bone marrow. Iron absorption is increased because of the expanded erythron causing high levels of GDF-15, ERFE or other proteins, with suppression of hepcidin production. A wide range of other problems are particularly associated with

thalassaemia intermedia, including pulmonary hypertension, hypercoagulability, pseudoxanthoma elasticum and other connective tissue disorders, hypersplenism, leg ulceration, folate deficiency, extramedullary haemopoietic tumour masses in the paraspinal regions and skull, gallstones and a marked proneness to infection. Many of these problems worsen with age, due to iron loading and cardiovascular impairment, and increasing numbers of patients become transfusion dependent. Because of the heterogeneity of these disorders, management is highly dependent on the clinical picture that evolves in an individual patient rather than any particular genotype or other biomarker; all patients should be followed up very carefully from early childhood. The haemoglobin constitution of β-thalassaemia intermedia depends on the contributing genotypes, and in many cases is similar to that found in the major forms.

Treatment

Intermittent blood transfusions are often necessary due to falls in haemoglobin caused by fever and infection, particularly with parvovirus (erythrovirus) B19, which causes reticulocytopenia. It can be difficult to decide who would benefit from more regular blood transfusions and when to start them. In countries with a ready supply of safe blood, there is an increasing tendency to start regular transfusions even in children, maintaining the haemoglobin at greater than 80 g/L in order to avoid the emerging complications of skeletal deformities, pulmonary hypertension and osteopenia. There is also some evidence that this improves the quality of life for many patients. This intensive approach is not possible in much of the world and management consists of reserving transfusion for severe symptomatic anaemia.

Even in the absence of regular erythrocyte transfusions, significant levels of iron accumulate through increased gastrointestinal absorption. Clinical trials with iron chelators have shown that it is possible to remove this excess iron safely, and this is increasingly practised (Chapter 12). However clinical complications of iron overload are relatively uncommon in thalassaemia intermedia, and it is less clear when chelation should be started.

Pharmacological treatment to increase HbF and total haemoglobin levels is potentially applicable to thalassaemia intermedia, in that relatively small increases in haemoglobin levels with a corresponding reduction in ineffective erythropoiesis could help a patient thrive who would otherwise require regular transfusions. Hydroxycarbamide (hydroxyurea) is the most widely used drug in this context, and while some patients undoubtedly benefit, in general, results are disappointing. Butyrate and other short-chain fatty acids also promote HbF synthesis and have been used with limited clinical success in thalassaemia intermedia. A number of newer drugs are being developed that may boost HbF to a greater extent, most notably the new generation of short-chain fatty acid derivatives and immunomodulatory drugs such as pomalidomide and lenalidomide. Thalidomide is quite widely used in some countries in combination with hydroxyurea to increase haemoglobin levels. It is currently unclear how useful luspatercept will be in managing these patients, but it is likely to have some role in high-income countries. Another promising approach for both alpha and beta thalassaemia is activating pyruvate kinase which increases glycolytic flux and reduces oxidative stress. Mitapivat and etavopivat are currently under clinical development. Gene therapy is available in some countries, but currently has a very small role to play in the management of thalassaemia, although it is likely to become increasingly important over the next decade.

Screening for thalassaemia

Preconception, antenatal and neonatal screening programmes are important in the clinical care and public health management of haemoglobinopathies. Thalassaemia is prevalent in many middle- and low-income countries, where either treatment is not available or the cost of regular blood transfusions and chelation is a major drain on limited medical resources. In other countries, including parts of Europe, Australia and North America, it is important to identify the antenatal risk of having a baby with thalassaemia to provide informed parental choice. Apart from haemopoietic stem cell treatments, there is no definitive treatment, and many countries in which the disease is common are putting a major effort into programmes for its prevention.

Premarital and preconception screening

Ideally, individuals know their thalassaemia status before they decide to have children, and potentially use this information to choose a suitable partner. Some programmes therefore concentrate on screening teenagers at school. Other countries insist that a couple are screened for thalassaemia status before they can get married; this latter approach has been successful in Cyprus and parts of the Middle East, such as Bahrain, where social pressures have reduced the number of at-risk partnerships.

Antenatal screening, prenatal diagnosis and preimplantation genetic diagnosis

Many screening programmes concentrate on identifying pregnant women who are thalassaemia carriers in the first trimester of pregnancy. This is done by varying combinations of blood tests and the identification of women at high risk of carrying thalassaemia based on their ethnic origin. The latter approach is potentially effective in areas with a low prevalence of thalassaemia in the native population, such as northern Europe, although increasing ethnic diversity is reducing the feasibility of such selective screening. If a woman is found to be a carrier, screening is then offered to her partner, and if both are carriers, they are counselled about the risk of the fetus inheriting a severe form of thalassaemia and offered prenatal diagnosis (PND).

PND of the thalassaemias was first carried out by fetal blood sampling, between 18 and 20 weeks of gestation, and was based on measuring relative rates of globin chain synthesis. Fetal blood sampling has now been replaced by fetal DNA analysis obtained at 11–12 weeks' gestation by chorionic villus sampling or amniocentesis. DNA analysis is now based largely on direct sequencing of the globin genes and specific techniques to detect large deletions, such as multiplex-ligation-dependent probe amplification. This produces rapid results, typically taking 48–72 hours, with accuracy of greater than 99%.

Couples can use this information to make an informed choice as to whether to continue the pregnancy if the fetus is affected. Some couples find PND helpful in preparing for the birth of a potentially ill child, even though they would not contemplate termination. The main complication of invasive PND is the increased risk of miscarriage of about 1%. This has led to research to develop non-invasive methods of PND based on maternal blood sampling. Maternal blood contains small numbers of fetal cells and also cell-free fetal DNA, both of which could potentially be used to diagnose fetal thalassaemia. The low concentration of fetal material relative to maternal has made this technically very difficult, although advances in methods of DNA analysis and sequencing platforms are starting to make this feasible.

Some couples want to avoid having a fetus with thalassaemia, but find PND and selective termination unacceptable. Preimplantation genetic diagnosis involves the use of *in vitro* fertilisation techniques to generate 5–15 embryos; at the eight-cell stage, one embryonic cell can be removed and tested for thalassaemia alleles; it is then possible to only implant embryos without thalassaemia. Whilst appealing, it is a difficult, stressful and expensive procedure, with fewer than 50% couples taking home a baby. Advances in reproductive biology and DNA technology are making this more applicable, and it is available in an increasing number of countries.

Neonatal screening

It is possible to detect the majority of babies with severe thalassaemia by neonatal testing, either on cord blood or blood taken directly from the baby. Many neonatal screening programmes involve testing dried blood spots on pieces of filter paper, usually made from a heel-prick blood sample when the baby is 5–7 days old. This blood spot is also used to screen for other serious neonatal disorders, including phenylketonuria, congenital hypothyroidism and cystic fibrosis. If haemoglobin analysis shows HbF only, with no HbA or other haemoglobin variants, it is likely that the baby has inherited a severe form of β-thalassaemia and may be transfusion dependent; less severe possibilities include thalassaemia intermedia and homozygosity for HPFH. Babies identified in this way can then be followed up closely rather than waiting until they present following a period of prolonged illness. Parents can also be tested and given advice concerning the risk to future pregnancies.

Table 14.3 Diseases due to structural haemoglobin variants.

Sickle cell disease
Sickle cell anaemia, with homozygosity for the sickle mutation (HbSS)
Compound heterozygosity for HbS with other β-haemoglobin variants (HbS/C, HbS/D Punjab, HbS/O Arab)
Compound heterozygosity for HbS with β-thalassaemia (HbS/β-thalassaemia)
Haemolytic anaemia
Unstable haemolytic variants
Congenital polycythaemia
High-oxygen-affinity haemoglobin variants
Congenital cyanosis
Low-oxygen-affinity haemoglobin variants
M haemoglobins
Hypochromic microcytic anaemia (thalassaemia phenotype)
Variants with inefficient synthesis due to alternative splicing, e.g. HbE
Lepore haemoglobins
Unstable chain termination variants, e.g. Hb Constant Spring
Drug-induced haemolysis
e.g. Hb Zurich

Structural haemoglobin variants related to thalassaemia

The unstable haemoglobin disorders

The unstable haemoglobin disorders are a group of rare inherited haemolytic anaemias that result from structural changes in the haemoglobin molecule, which cause its intracellular precipitation with the formation of Heinz bodies (Table 14.3). Their true incidence is not known and there have been several well-documented instances in which patients with one of these variants have had no affected relatives, suggesting that they have arisen by a new mutation. Unlike more common globin gene mutations, there is no obvious ethnic or geographical variation. Haemoglobin Koln (β98, Val→Met) is perhaps the most common.

Molecular pathology and pathogenesis

Most of the unstable haemoglobins result from single amino acid substitutions or small deletions. For example, substitutions in or around the haem pocket can disrupt its anatomy and allow in water, with subsequent oxidative damage to haem, leading to precipitation of haemoglobin. Some substitutions, such as those involving proline residues, cause a marked disturbance of the secondary structure of globin chains. A few variants result from deletions of either single amino acids or several residues. For example, in Hb Gun Hill, five amino acids are missing,

including the haem-binding site. As the unstable haemoglobins precipitate in the red cells or their precursors, they produce intracellular inclusions (Heinz bodies) which, together with oxidant damage to their membranes, make the cells more rigid and hence cause their premature destruction in the microcirculation.

Clinical features

Unstable haemoglobins are characterised by a chronic, non-spherocytic haemolytic anaemia and splenomegaly. Like all chronic haemolytic anaemias, there is an increased incidence of pigment gallstones with their associated complications; the risk is particularly high if there is coinheritance of Gilbert syndrome (polymorphic variant in the promoter of the *UGT1A1* gene). The condition may become worse during periods of infection causing fever, and, in the more severe forms, such episodes are associated with life-threatening anaemia and the need for blood transfusion. Some oxidant drugs may increase the rate of haemolysis, and parvovirus (erythrovirus) B19 infection may cause temporary reticulocytopenia. Apart from icterus and splenomegaly, there are no characteristic physical findings.

Laboratory diagnosis

The peripheral blood film shows typical features of haemolysis, but the red cell morphology may be normal. Occasionally, there is mild hypochromia and microcytosis. Supravital staining with dyes such as methyl violet shows Heinz bodies in the peripheral blood, particularly after splenectomy. The most characteristic feature of the unstable haemoglobins is their heat instability. If a dilute haemoglobin solution is heated at 50 °C for 15 min, the unstable haemoglobins precipitate as a dense cloud. A similar effect can be induced by isopropanol at lower temperatures. The isopropanol heat sensitivity test is more sensitive and specific than simple heat instability. Some of these variants can be seen on haemoglobin electrophoresis, but others are electrophoretically silent, because they result from a neutral amino acid substitution; these can be demonstrated only by the heat precipitation test. DNA analysis can provide more definitive diagnosis, typically by sequencing of the α- or β-globin genes, and this is increasingly used at an early stage as sequencing gets faster and cheaper.

Treatment

Splenectomy seems to be beneficial in some cases, although experience is inevitably limited. Intermittent blood transfusions may be necessary. If haemolysis is very severe, the patient may benefit from regular blood transfusions and bone marrow transplantation should be considered.

High-oxygen-affinity haemoglobin variants

Some haemoglobin variants cause increased oxygen affinity, which results in varying degrees of polycythaemia. These are rare and occur sporadically; haemoglobin Chesapeake (α92, Arg→Leu) was the first one identified in 1966.

Molecular pathology

Some high-oxygen-affinity haemoglobin variants result from single amino acid substitutions at critical parts of the haemoglobin molecule that are involved in the configurational changes which underlie haem–haem interaction and the production of a sigmoid oxygen dissociation curve at the junctions between the α- and β-subunits. Others involve the amino acids concerned with the binding of 2,3-DPG to haemoglobin. Reduced 2,3-DPG binding moves the oxygen dissociation curve to the left, reducing the P_{50} and causing the haemoglobin to hold on to oxygen more avidly than normal. This causes functional anaemia, with tissue hypoxia, which in turn causes an increased output of erythropoietin and an elevated red cell mass.

Clinical features

Most affected individuals are completely healthy and are identified only when a routine haematological examination shows an unusually high haemoglobin level or haematocrit. There is no splenomegaly and, apart from a raised red cell mass, there are no associated haematological findings. Although it might be expected that a high-oxygen-affinity haemoglobin would cause defective oxygenation of the fetus, none of the reported families has a history of such problems.

Diagnosis

The condition should be suspected in any patient with a pure red cell polycythaemia associated with a left-shifted oxygen dissociation curve. Erythropoietin levels will typically be inappropriately high for the haematocrit. The absolute reticulocyte count may be slightly elevated, although the reticulocyte percentage should be normal. The erythron is expanded, as shown by high plasma concentrations of soluble transferrin receptor. The diagnosis can be confirmed by haemoglobin analysis, mass spectrometry, and increasingly DNA analysis.

Treatment

In asymptomatic individuals, no treatment is necessary. The difficulty arises if there is associated vascular disease, particularly coronary or cerebral artery insufficiency. As these patients require a high haemoglobin level for oxygen transport, venesection should be carried out with caution. Venesection is undertaken because of increased risk of vascular complications, and

typically the aim is to keep the haematocrit below 0.55, although there is little evidence to support this.

Low-oxygen-affinity haemoglobin variants

More than 50 haemoglobin variants with reduced oxygen affinity have been identified, often associated with other abnormal properties such as instability. The first to be described, Hb Kansas (β102 Asn→Thr), was found in a mother and son with unexplained cyanosis. The subjects were asymptomatic and had normal haemoglobin levels without any evidence of haemolysis. Like many of the high-affinity haemoglobins, the amino acid substitution in this variant was at the interface between the α- and β-globin chains. For reasons that are not clear, some substitutions in this region give rise to variants with a relatively low oxygen affinity. This condition should be thought of in any patient with unexplained congenital cyanosis.

Congenital methaemoglobinaemia due to haemoglobin variants

Several α- and β-globin variants associated with methaemoglobinaemia have been discovered. These are usually referred to as M-haemoglobins, for example haemoglobin M-Boston (α58, His→Tyr). These disorders, unlike the genetic methaemoglobinaemias due to enzyme defects, follow a dominant pattern of inheritance. The patients are blue in colour and may have mild polycythaemia as methaemoglobin does not carry oxygen. Diagnosis is based on detecting high methaemoglobin levels, which are increasingly measured by blood gas analysers and some pulse oximeters. Increased methaemoglobin levels should be confirmed on formal laboratory analysis using spectrophotometry. Subsequently haemoglobin analysis using electrophoresis or mass spectrometry is performed, or sequencing of the α- and β-globin genes.

Acknowledgement

Parts of the present chapter are based on the corresponding previous edition chapter, and for this, we acknowledge the contribution of the late Professor Sir David Weatherall.

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CHAPTER 15

Sickle cell disease

15

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Introduction

Sickle cell disease (SCD) is an inherited chronic haemolytic anaemia whose clinical manifestations arise from the tendency of the haemoglobin (HbS or sickle haemoglobin) to polymerise and deform red blood cells into the characteristic sickle shape. This property is due to a single nucleotide change in the β-globin gene leading to substitution of valine for glutamic acid at position 6 of the β-globin chain ($\beta^{6glu \rightarrow val}$ or β^{s}). The homozygous state (HbSS or sickle cell anaemia) is the most common form of SCD, but interaction of HbS with thalassemia and certain variant haemoglobins also leads to sickling. The term 'sickle cell disease' is used to denote all entities associated with sickling of haemoglobin within red cells (Table 15.1).

Geographic distribution of sickle mutation

Several distinct β-globin gene haplotypes are associated with the sickle mutation, and their distribution provides evidence for origin of the mutation in several locations within Africa (the Senegal, Benin and Bantu haplotypes) and Asia (the Arab–Indian haplotype). The sickle trait bestows survival benefits in areas endemic for *falciparum* malaria, and the distribution of SCD historically paralleled this disease. The sickle haemoglobin-containing red cells inhibit proliferation of *P. falciparum* and are more likely to become deformed and removed from the circulation. In recent times, the dissemination of the sickle mutation in different areas of the world took place from the movement of populations via trade routes and the slave trade (Table 15.2). The prevalence of SCD varies tremendously amongst ethnic and tribal groups within a geographic area. The ethnic groups represented in the California newborn population during a 3-year interval showed that 93% of sickle cell anaemia cases (HbSS) was reported in Black newborns. However, a large proportion of HbS/β-thalassaemia cases (12.5%) was observed in the Hispanic population.

Pathophysiology

Molecular basis of sickling

Deoxygenation of HbS leads to a conformational change that exposes a hydrophobic patch on the surface of the β^{s}-globin chain at the site of β^{6} valine (Figure 15.1). Binding of this site to a complementary hydrophobic site on a β-subunit of another haemoglobin tetramer triggers the formation of large polymers. The polymers consist of staggered haemoglobin tetramers that aggregate into 21-nm diameter helical fibres, with one inner and six peripheral double strands. The polymerisation proceeds after a delay, the length of which is extremely sensitive to the intracellular deoxy-HbS concentration. Even a small increase in deoxy-HbS concentration, such as might occur with cellular dehydration, profoundly shortens the delay time and augments sickling. The haemoglobin tetramers first aggregate into a nucleus, which rapidly expands into a fibre. The newly formed fibre provides nuclei on its surface for aggregation of haemoglobin tetramers to form several more fibres.

The polymerisation of HbS in the circulating red cells is influenced by the oxygenation status, the intracellular haemoglobin concentration and the presence of non-sickle haemoglobins. Acidosis and elevated levels of 2,3-diphosphoglycerate (2,3-DPG) promote polymer formation by reducing the oxygen affinity of haemoglobin. The presence of HbA within the red

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 15.1 The sickling syndromes.

Genotype	Mean haemoglobin (g/L)	Haemoglobin electrophoresis/HPLC (%)					
		MCV	S	A	F	A_2	Other
SS	81	N	80–95	–	2–20	N	–
SS –α/αα, SS –α/–α	86, 92	↓, ↓	80–90, 80–90	–, –	2–20, 2–20	3.3–3.8, 3.3–3.8	–, –
SC	110	↓	40–50	–	1–4		C: 40–50
S/β^0 -thalassaemia	88	↓	75–90	–	2–20	4–6	–
S/β^+ -thalassaemia	115	↓	50–85	5–30	2–20	4–6	–
SD Punjab	82	N	40	–	2.5–5	2–3	D Punjab: 50
SO Arab	81	N	45	–	4–7		O Arab: 45
S Lepore	110	↓	75	–	3.5–40	2	Lepore: 10
SE	130	↓	60	–	4		E: 30–35
S/HPFH	137	N or ↓	60–70	–	25–35	1.5–2.5	–
AS*	N	N	30–45	50–65	2–5	N	–

MCV, mean corpuscular volume; HPLC, haemoglobin high-performance liquid chromatography.
* Sickle cell trait is asymptomatic.

cells, as in sickle trait, inhibits polymerisation by diluting HbS. The inhibitory effect of fetal haemoglobin, HbF, on polymerisation of HbS is more profound owing to the greater amino acid disparity between the β^s and γ-globin chains.

Effect on erythrocytes

Red cells acquire the sickle or elongated shape upon deoxygenation as a result of intracellular polymerisation of HbS, a phenomenon that is reversible on reoxygenation. Even in the normally shaped red cells, however, the presence of HbS polymer reduces deformability, with consequent increase in blood viscosity. HbS has reduced oxygen affinity compared with HbA which further exacerbates HbS polymerisation, further decreasing oxygen affinity. In addition, 2,3-diphosphoglycerate (2,3-DPG), a glycolytic intermediate, is physiologically present at very high levels in sickle erythrocytes and reduces Hb oxygen affinity even further.

Table 15.2 Areas of high prevalence of sickle mutation.

Geographic region	Heterozygote rate (%)
Africa	
Northern	1–2
Western	10–30
Central	7–37
Southern	0–5
Mediterranean	
Northern Greece	1–27
Southern Italy	1
Americas	
United States: African ancestry	8
Caribbean: African ancestry	10
Brazil: non-white	7
Asia	
Saudi Arabia: south-west	5
Saudi Arabia: eastern province	25
India: central India–tribal population	20–30

Haemolysis

Repeated or prolonged sickling progressively damages the red cell membrane, which is a phenomenon of primary importance in the pathophysiology of SCD and leads to haemolysis. The red cell survival, measured by ^{51}Cr assay, is 4–25 days, with dense cells surviving for a considerably shorter time than red cells containing some HbF (F cells). Patients have greatly expanded bone marrow space, but the serum erythropoietin level is lower than expected for the extent of anaemia because of the decreased oxygen affinity of HbS. Individuals with concomitant deletion of one or two α-globin genes, or the Senegal or Arab–Indian haplotypes, have higher baseline haemoglobin levels.

Oxidised erythrocyte membrane proteins reveal antigens that bind to existing antibodies and membranes expose phosphatidylserine (PS). Both these processes promote phagocytosis of erythrocytes by macrophages, a pathway of *extravascular haemolysis. Intravascular haemolysis* of erythrocytes releases two factors that interfere with nitric oxide synthase (NOS) activity. The enzyme *arginase 1* competes with NOS for L-arginine, the substrate required for NO production by NOS. This process facilitates cell proliferation and vascular remodelling. *Asymmetric dimethylarginine* (ADMA) is an endogenous NOS inhibitor and is abundant in erythrocytes and is released during

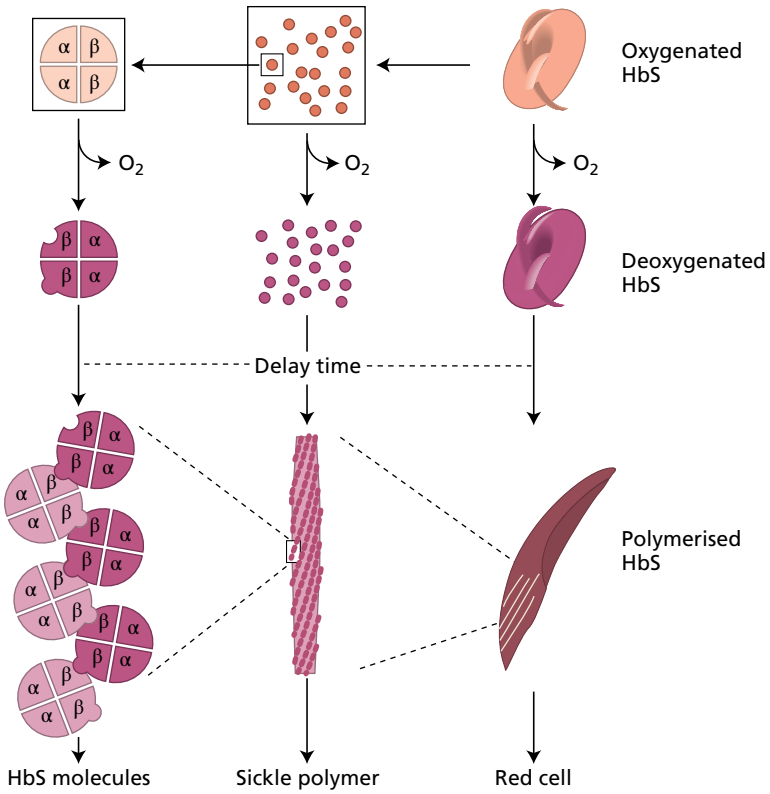

Figure 15.1 Induction of red cell sickling. As red cells traverse the microcirculation, oxygen is released from oxy-HbS (red circles), generating deoxy-HbS (purple circles). Conformational change exposes a hydrophobic patch at the site of the β^6 -valine replacement, shown as a projection (left column), which can bind to a complementary hydrophobic site on a subunit of another haemoglobin tetramer, shown as an indentation. Only one of the two β^6 -valine sites in each HbS tetramer makes this contact. The middle column shows the assembly of deoxy-HbS into a helical 14-strand fibre, shown as a twisted rope-like structure. The delay time is inversely proportional to the intracellular haemoglobin concentration raised to the 15th power. As deoxy-HbS polymerises and fibres align, the red cell is distorted into an elongated banana or 'sickle' shape (right column) (Source: Bunn, 1997 [*New Engl J Med* **337**: 762–9]. Reproduced with permission of Massachusetts Medical Society.)

haemolysis. Both ADMA and depletion of L-arginine by arginase 1 could contribute to uncoupling of NOS, which then produces reactive oxygen species (ROS) instead of NO. ROS further depletes NO, leading to vasoconstriction and vascular remodelling, especially in the lung. NO deficiency promotes platelet activation and activation of blood clotting proteins.

Vaso-occlusion

Several processes contribute to development of vaso-occlusion in SCD. Slowing of blood flow arises from abnormal regulation of vascular tone as a result of diminished nitric oxide (NO)-induced vasodilatation. This is aggravated by increase in blood viscosity, resulting from less deformable red cells, a phenomenon called abnormal rheology. Vaso-occlusion is initiated by adhesion of young deformable red cells to the vascular endothelium and is followed by trapping of rigid irreversibly sickled cells. In postcapillary venules, activated endothelial cells that express P-selectin and E-selectin can bind rolling neutrophils. Haemolysis activates the innate immune system including monocytes, macrophages and cytokines which promote an inflammatory state and activate endothelial cells promoting platelet and neutrophil adhesion and formation of neutrophil extracellular traps (NETs). (Figure 15.2). Even reticulocytes and deformable erythrocytes (i.e. erythrocytes that have not become permanently sickled) are substantially more adhesive than the irreversible and dense sickle erythrocytes. Platelets play an important part in the pathophysiology of SCA and are in an activated state, with high levels of P-selectin and activated αIIbβ3 integrin. Platelets are found in the circulating heterocellular aggregates of neutrophils and red blood cells (mainly reticulocytes) in the blood from individuals with SCA. Platelets could also act as accessory cells of the innate immune system by releasing cytokines.

Clinical manifestations

Clinical symptoms vary tremendously between patients with SCD. The disease is more severe in patients with HbSS or HbS/β^0-thalassaemia than in those with HbS/β^+ -thalassaemia

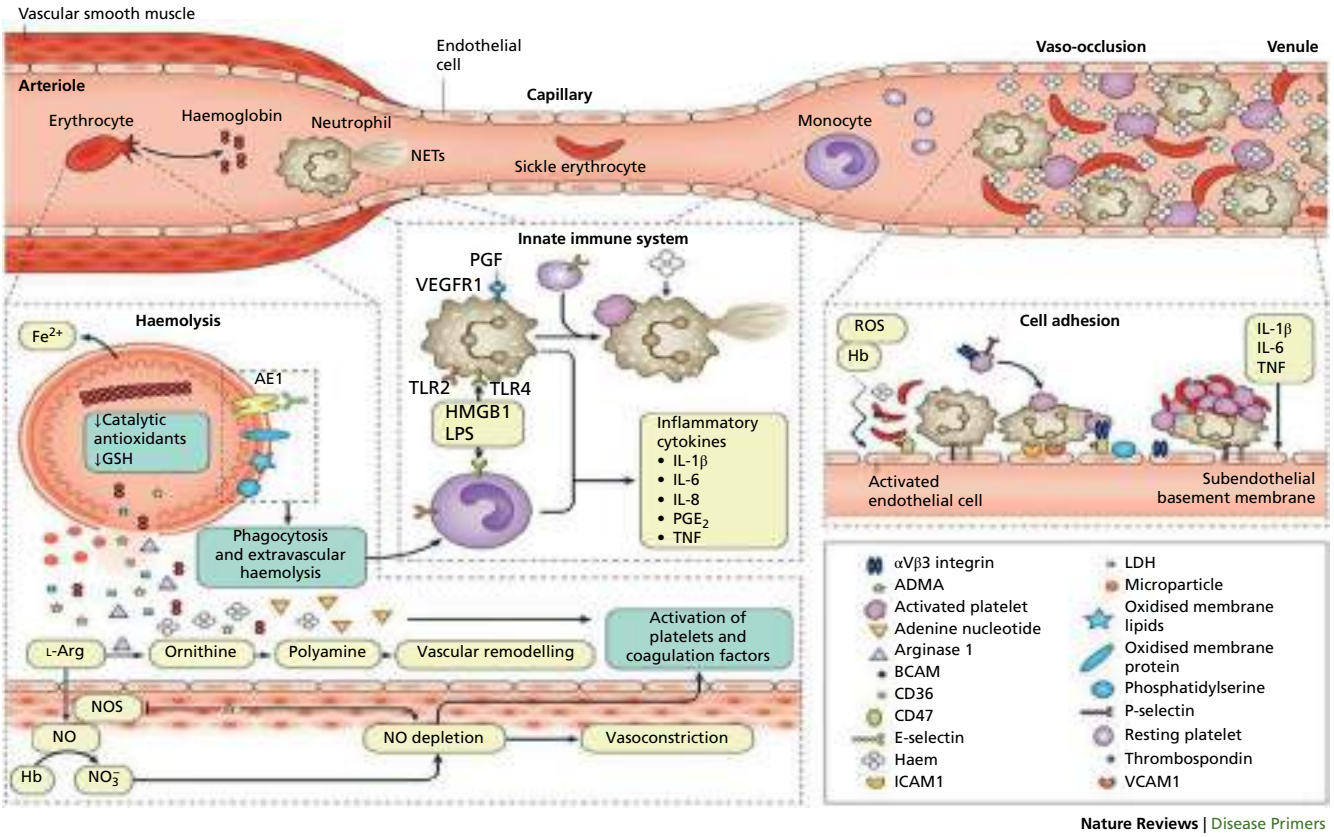

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Figure 15.2 Mechanisms of erythrocyte haemolysis, vaso-occlusion, cell adhesion and immune system activation in sickle cell disease.

or HbSC disease. However, for increasingly recognised reasons, the disease severity varies enormously, even within the subgroup of patients with HbSS. Genetic modifiers that influence both laboratory and clinical parameters in part explain the phenotypic heterogeneity of SCD. Some of the genetic modifiers known to impact disease phenotype include coinheritance of α-thalassaemia trait, β-globin haplotype, modifiers of endogenous haemoglobin F production, *UGT1A1* promoter polymorphisms and genes that have shown protection against stroke. The coinheritance of one or two α-gene deletions results in lower MCV, MCH, total bilirubin and absolute reticulocyte counts. The Senegal and Arab–Indian β-globin haplotypes produce less severe clinical disease than the other African haplotypes. The high HbF level observed in hereditary persistence of fetal haemoglobin (HPFH) is associated with very mild disease. Certain *BCL11A* single nucleotide polymorphisms known to positively affect endogenous fetal haemoglobin production have been shown to have favourable laboratory profiles and may serve as potential targets for gene editing of haemopoietic stem cells. Specific promoter mutations within the *UTG1A1* gene as seen in Gilbert syndrome, encoding an enzyme necessary for bilirubin conjugation, are correlated with increased bilirubin levels and a propensity to develop cholelithiasis. Mutations within the *GOLGB1* and *ENPP1* genes have been shown to have a protective effect against stroke in SCD. These and other genetic modifiers help to explain the phenotypic heterogeneity observed in this monogenic disease. Genome-wide genetic association studies and identification of epigenetic phenomena are likely to identify other potential modifiers of disease severity.

In countries with inadequate healthcare, SCD is associated with high mortality in the first 3 years of life as a result of sepsis and splenic sequestration. In the developed world, the typical patient with SCD has moderately severe anaemia, leads a relatively normal life interrupted by 'crises' as a result of vaso-occlusion, and has a life expectancy of over 45 years.

Anaemia

The underlying β-globin genotype primarily determines the baseline haemoglobin value in SCD, but exacerbation of anaemia can occur for numerous reasons. Patients with more severe anaemia at baseline have a greater probability of developing stroke and renal dysfunction. On the other hand, a higher haemoglobin level is associated with a higher incidence of painful episodes, avascular necrosis and acute chest syndrome. Infants with SCD have lower than normal haemoglobin levels after the

neonatal period, and the decline continues until it reaches a nadir between 12 and 15 months of age. Boys are slightly more anaemic than girls in the first decade, whereas adult men have higher haemoglobin values than women. Gradual exacerbation of anaemia is observed in both sexes beginning in the fifth decade.

A gradual decrease in haemoglobin level from the baseline value may indicate an underlying folate or iron deficiency. In older patients, however, inadequate erythropoietin production due to chronic renal insufficiency is the most important aetiology of worsening anaemia. Many such patients will become transfusion dependent, although recombinant human erythropoietin therapy can improve the anaemia.

Acute exacerbations of anaemia are observed with aplastic crises and splenic sequestration. The transient arrest of erythropoiesis and the resultant reticulocytopenia in aplastic crisis is most often due to parvovirus (erythrovirus) B19 infection. The reticulocytopenia begins 5 days after exposure, lasts for 7–10 days and is followed by recovery with reticulocytosis and normoblasts in peripheral blood. Blood transfusion is often required in the short term. Parvovirus B19 infection is followed by development of lifelong protective immunity.

Splenic sequestration is a serious complication in young children whose spleen has not yet undergone fibrosis due to recurrent vaso-occlusion. The peak incidence of the first episode of sequestration is between 6 and 12 months of age and affects 30% of all patients. Approximately 15% of patients die during the acute episode and the condition recurs in half of the survivors. Episodes may be triggered by a viral illness and the rapid acute enlargement of the spleen traps a significant proportion of the blood volume. Clinically, the child presents with acutely worsening anaemia (>20 g/L fall in haemoglobin), reticulocytosis, enlarging spleen and hypovolaemic shock. Prompt restoration of the blood volume and correction of anaemia is required. Splenectomy is recommended following a sequestration crisis that is life-threatening or for recurrent splenic sequestration episodes. Chronic transfusion therapy is sometimes used in infants with life-threatening anaemic episodes. Parent education to detect splenic enlargement and seek early medical attention significantly reduces the risk of death from sequestration crisis.

Acute painful episode

An acute episode of pain due to vaso-occlusion is the most frequent symptom for which patients with SCD seek medical attention. Painful episodes are more common in young adults and tend to diminish in older patients. One-third of patients with SCD rarely experience pain, whereas a small subgroup of patients suffer from recurrent episodes. When patients maintain a pain diary, painful events are noted on up to half of the days, but are not severe enough to require visit to a physician. Painful episodes vary in intensity and generally last for a few days. The majority of the episodes have no identifiable cause, although some attacks are precipitated by cold, dehydration, infection, stress or menses. There are no physical or laboratory findings that can determine whether a patient with SCD is having a pain episode or not.

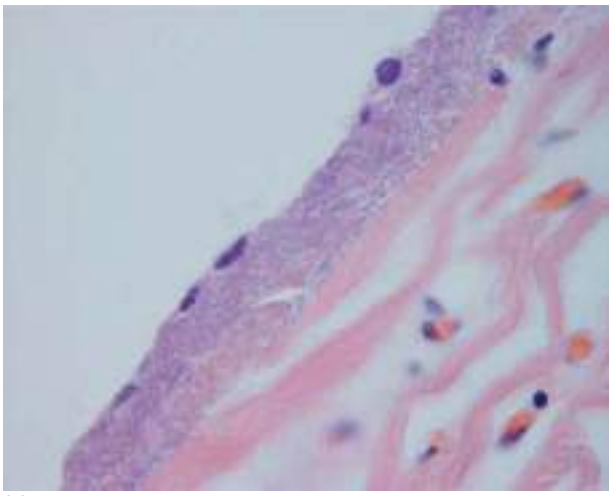
In young children, the initial pain episode typically presents as dactylitis or hand–foot syndrome, with swelling over the dorsal surface of hands and feet. It arises from bone infarction affecting the small bones and the swelling subsides over 1–2 weeks. The radiographs show thinning of cortex and destructive changes of the affected small bones several weeks after onset. In older children and adults, the common sites of pain are the back, chest, extremities and abdomen. Chest pain is of special significance as it can precede development of acute chest syndrome. Frequent incapacitating painful episodes that are inadequately managed have adverse psychosocial consequences and stress the physician–patient relationship.

Growth and development

Children with SCD are born with normal weight but fall behind other children by the end of the first year. The weight deficit persists through adulthood and imparts a thin habitus to the typical patient, although obesity is seen in some cases. The rate of growth is lower than normal in SCD patients, and the pubertal growth spurt is delayed by 1–2 years, but the final adult height is normal. Delays also occur in skeletal maturation and onset of puberty, and female patients achieve menarche 1–2 years later than their peers.

Infections

Early loss of splenic function from recurrent vaso-occlusion and the inability to make specific IgG antibodies to polysaccharide antigens increases the risk of fulminant sepsis. Pneumococcal infection is a serious problem in SCD, particularly in children under 3 years (Figure 15.3). Meningitis can accompany pneumococcal sepsis, and the overall mortality rate is 20–50%. Patients who have had previous pneumococcal sepsis are at increased risk for recurrent episodes and must remain on lifelong penicillin prophylaxis. *Haemophilus influenzae* type B is the next most common organism and affects older children. There is considerable variation in the relative incidence of bacterial organisms causing sepsis in young children with SCD in various regions of the world. In Africa, *Salmonella* spp., *Klebsiella* spp., *Escherichia coli* and *Staphylococcus* spp. are more commonly isolated from the blood of febrile children than *Streptococcus pneumoniae*. The incidence of pneumococcal and *H. influenzae* sepsis has declined as a result of penicillin prophylaxis and vaccination of infants. The risk of death during septic episodes has decreased considerably owing to empirical use of antibiotics to treat fever in SCD.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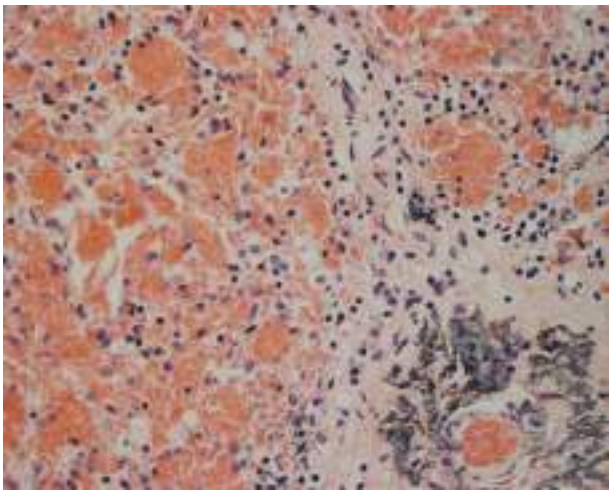
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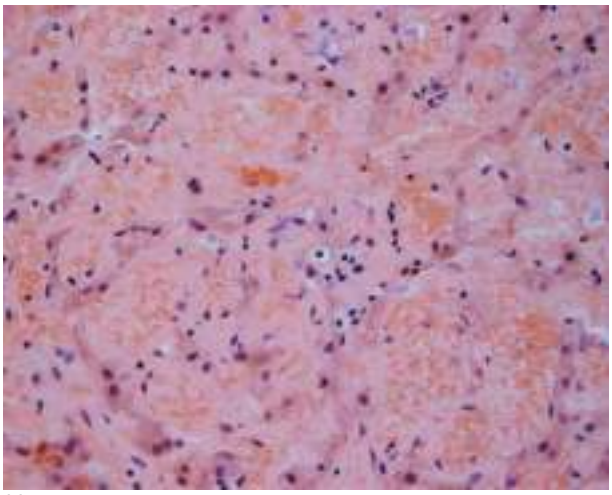

(c)

Figure 15.3 Overwhelming pneumococcal sepsis in a 7-year-old child. (a) Numerous bacteria in the blood adjacent to the right ventricular wall. Massive sequestration of the spleen (b) and the liver (c).

Of the other infections, pneumonia is particularly common in SCD and can be difficult to differentiate from non-infective causes of acute chest syndrome. The most frequent organisms responsible for pneumonia are *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *S. pneumoniae* and *H. influenzae*. Lung infections can also arise due to respiratory viruses. In adults, bacteraemia and urinary tract infections due to *E. coli* and other Gram-negative organisms are more frequent. Patients with SCD are susceptible to osteomyelitis caused by bone infarction resulting from vaso-occlusion. The infection is typically due to *Salmonella* spp. or *Staphylococcus aureus*.

Recently COVID-19 infections were shown to have a high mortality in sickle cell patients especially those with underlying chronic kidney disease, pulmonary hypertension, congestive heart failure and history of stroke. Children with SCD with history of pain and renal and heart or lung problems were shown to have worse outcomes with COVID-19 infection. Patients presented with vaso-occlusive pain during the pandemic and several of them developed acute chest syndrome.

Neurological complications

Stroke, silent cerebral infarcts (silent strokes), and cognitive morbidity are the most common permanent sequelae of SCD. The risk of stroke is increased with lower baseline haemoglobin, low HbF level, high leucocyte count or high systolic blood pressure. A large prospective cohort study demonstrated that by 40 years of age, ~20% and ~10% of adults with phenotype haemoglobin SS (HbSS) or HbSC had a cerebrovascular accident respectively. For most children with HbSS living in low-middle-income settings, ~11% will have a stroke by 18 years of age. Stroke due to infarction is more frequent in young children and those over 30 years of age, whereas haemorrhage is more common between 20 and 30 years. Stroke is rare in infants, increases to 1 in 100 patients per year between 2 and 9 years, and then diminishes to half that incidence in older patients. The incidence of stroke is 5–10 times greater in HbSS compared with HbSC, HbS/β^+ -thalassaemia or HbS/β^0 thalassaemia.

Focal seizures or transient ischemic attacks are common presenting symptoms of overt stroke, followed by hemiparesis, coma and speech or visual disturbances. However, the most common cause of permanent neurological injury in children and adults with HbSS and HbSβ^0 thalassaemia is a silent cerebral infarct, occurring in ~39% of children by age 18 years of age and >50% of adults by age 30 years of age. Both stroke and silent cerebral infarcts are associated with significant cognitive impairments that may significantly alter educational attainment, employment status and quality of life. The site of bleeding in haemorrhagic stroke is frequently subarachnoid, and these patients present with severe headache, vomiting and coma. Death can occur during the acute event, particularly with haemorrhagic stroke.

Patients with neurological symptoms should be evaluated initially with a computed tomography (CT) to rule out

haemorrhage and acute mass effect followed by magnetic resonance imaging (MRI) as CT alone is not sensitive for ischaemic stroke. Immediate exchange transfusion to lower the HbS level to less than 30% is required. Patients with haemorrhage may require surgical intervention to ligate accessible aneurysms.

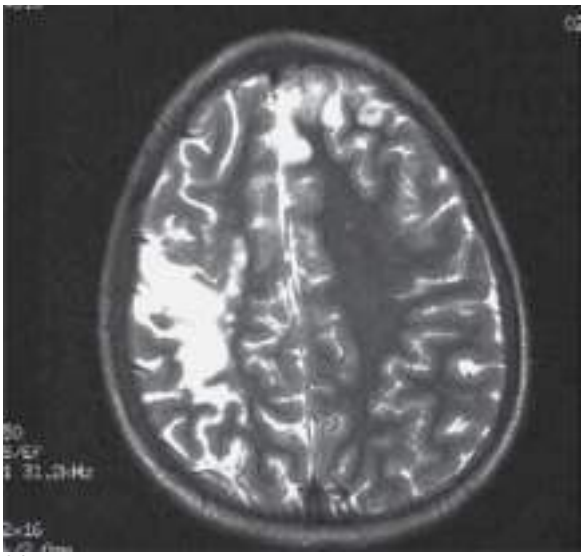
Moyamoya disease in non-SCD patients is a rare progressive cerebrovascular disorder caused by blocked arteries at the base of the brain in the basal ganglia. The name 'moyamoya' means 'puff of smoke' in Japanese and describes the look of the tangle in tiny vessels formed to compensate for the blockage. Vascular damage in SCD results from elevated cerebral blood flow velocities and interaction of rigid or adherent sickle cells with the vessel wall. Angiography demonstrates stenosis or occlusion of vessels in the circle of Willis and internal carotid arteries, sometimes with aneurysm formation or development of moyamoya disease (Figure 15.4b). Surgical vascular bypass procedure with extracranial arteries (encephalodural synangiosis) should be considered in children with SCD and moyamoya disease.

Prevention, early detection and treatment of neurologic complications are important. Transcranial Doppler (TCD) ultrasonography has been shown to be an effective screening tool for identification of stroke risk. TCD screening should begin at 2 years of age and continue annually into the late teenage years up to a minimum of 16 years of age. Increased blood flow velocity due to stenosis can be detected by TCD ultrasonography in asymptomatic patients, and flow rates in excess of 200 cm/s correlate with a high risk of stroke. Development of first stroke in children at risk, who are identified by elevated cerebral Doppler blood flow velocity, can be prevented effectively through regular transfusions. A randomised controlled trial (TWiTCH) comparing the effectiveness of hydroxycarbamide as an alternative to transfusion for primary stroke prevention in 2016 showed that for high-risk children with sickle cell anaemia and abnormal TCD velocities, after 4 years of transfusions and without severe MRA vasculopathy, hydroxycarbamide therapy can substitute for chronic transfusions to maintain TCD velocities and help prevent primary stroke. In patients who have already had a stroke, a prospective randomised controlled trial comparing transfusion to hydroxycarbamide for secondary stroke prevention was undertaken (SWiTCH trial) but was terminated early due to a high prevalence of events in the hydroxycarbamide arm. Patients treated with transfusions had fewer neurologic as well as non-neurologic sickle-cell-related serious adverse events. Though hydroxycarbamide has been shown to lower the incidence of pain and acute chest syndrome, its efficacy in the prevention and progression of cerebral vascular disease is less effective. Blood transfusions are therefore still considered the standard of care for secondary stroke prevention and initial therapy for primary stroke prevention in patients with abnormal TCD velocities. In a recent randomised single-blind clinical trial of children with silent cerebral infarcts (SITS trial), regular blood transfusion therapy significantly reduced the incidence of the recurrence of cerebral infarcts in children with sickle cell anaemia.

Cognitive impairment is very common amongst children and adults with sickle cell anaemia. In the most comprehensive meta-analysis to date, children with HbSS with normal MRIs of the brain, silent cerebral infarct and strokes had a mean full-scale intelligence quotient (FSIQ) of 91, 84 and 73 respectively. Few comprehensive cognitive studies in adults with sickle cell anqemia have been done that include assessment of FSIQ, employment status and assessment of cerebral infarct status. Optimal strategies to screen and implement rehabilitation for cognitive impairment have not been established for sickle cell anaemia. The use of formal screening tools improves the ability to detect developmental delays or cognitive impairment compared with informal observational methods. Identification of developmental or cognitive delays increases likelihood of access to remediation services. Vascular cognitive impairment is of particular concern in adults with SCD and requires ongoing efforts to identify cognitive symptoms and medical risk factors to help patients and families understand the cause of functional deficits and discuss the prognosis to plan for future needs. Outpatient services such as speech therapy, occupational therapy or rehabilitation psychology may be indicated and available depending on the results of a full evaluation.

Pulmonary complications

Acute and chronic pulmonary complications are the leading cause of death in older patients. The acute chest syndrome is characterised by hypoxia, tachypnoea, fever, chest pain and pulmonary infiltrate on chest radiography (Figure 15.4c). Acute chest syndrome often follows a painful event, particularly in adults (Table 15.3). The pathogenesis of acute chest syndrome involves vaso-occlusion, infection or embolisation of bone marrow fat. Infections due to *Mycoplasma*, *Chlamydia*, *Legionella*, pneumococcus, *H. influenzae* and other viruses are more likely in children. Recently, COVID-19 illness was shown to occur more frequently in children if they had frequent vaso-occlusive events prior to the infection or underlying heart and lung disease related to SCD. Fat-laden pulmonary macrophages in the airways due to fat embolisation from the bone marrow are present in half of the cases. Hypoxia due to acute chest syndrome can result in widespread sickling and vaso-occlusion, with risk of multiorgan failure. Patients should receive supplemental oxygen, incentive spirometry and antibiotic therapy directed towards the common organisms. Most patients have a bronchoreactive component and should receive bronchodilator therapy. Patients require close monitoring for persistent hypoxia or worsening lung consolidation for which blood transfusion should be provided. Exchange transfusion and mechanical ventilation are sometimes needed in rapidly progressive cases. NO and steroids may be beneficial in life-threatening cases.


(a)

(b)

(c)

(d)

(e)

Figure 15.4 Complications of sickle cell disease: (a) Stroke; (b) Moyamoya transformation; (c) Acute chest syndrome; (d) Avascular necrosis of hip joint; (e) Chronic leg ulcer.

Table 15.3 Presenting symptoms of acute chest syndrome.

Symptom	Children (%)	Adults (%)
Fever	86	70
Shortness of breath	31	58
Chest pain	27	55
Extremity pain	22	58
Rib pain	14	30

Adults are more likely than children to have pain preceding the onset of pulmonary symptoms.

Chronic pulmonary problems seen in SCD are obstructive and restrictive lung disease, pulmonary hypertension and hypoxia. Asthma, a form of obstructive lung disease, has a multifactorial pathophysiology in SCD and is influenced by genetic factors, environmental factors, the presence of a chronic underlying inflammatory state, and haemolysis-induced arginine–nitric-oxide dysregulation. Asthma has been shown to be an independent predictor of morbidity and mortality and may be missed during a physical examination. Screening for asthma with formal pulmonary function testing should be considered. Sleep-disordered breathing, diagnosed by formal sleep polysomnography, may have significant consequences for outcomes in children and potentially, adults with SCD. Sleep-disordered breathing is defined by abnormal respiratory patterns such as apnoeas and/or hypopnoeas and hypoventilation during sleep resulting in daytime sleepiness or fatigue that interferes with an individual's ability to function and reduces quality of life. Despite its utility, polysomnography as a screening tool for asymptomatic individuals with SCD is not clear but should be considered for symptomatic patients.

Pulmonary hypertension is another independent predictor of mortality and morbidity in SCD. The diagnosis of pulmonary hypertension is associated with a 10-fold increase in relative risk of dying compared with patients who have normal pulmonary artery systolic pressure. Echocardiography can detect an elevated tricuspid regurgitant velocity (>2.5 m/s), which is observed in 20% of paediatric patients. Right heart catheterisation is necessary to confirm the diagnosis (pulmonary artery pressure >25 mmHg). The prevalence of right heart catheter-confirmed pulmonary hypertension is approximately 10%. Pulmonary hypertension is more frequent amongst patients with high rates of chronic haemolysis, reflected by marked elevation in plasma lactate dehydrogenase (LDH). Disease-modifying therapeutics and/or chronic transfusion therapy should be strongly considered in SCD patients with pulmonary hypertension. Severely affected patients have been treated with regular transfusions, anticoagulation and oxygen inhalation. Current data do not support the use of targeted pulmonary arterial hypertension pharmaceuticals in SCD unless obstructive sleep apnoea, significant lung disease and left heart failure have been ruled out. A pulmonary hypertension expert must be involved in the patients' care if there are concerns for pulmonary hypertension. Lastly, hypoxia, both daytime and nocturnal, has been associated with the development of pain and acute chest syndrome and can be prevented with effective treatment.

Hepatobiliary complications

The liver can be affected by hepatic sequestration, intrahepatic cholestasis, transfusion-acquired infection and transfusional haemosiderosis. Episodes of cholestasis due to intrahepatic sickling can lead to liver failure in rare instances. Pigmented gallstones are seen in two-thirds of patients, particularly those with HbSS, and can occur in young children. Patients with abdominal symptoms attributable to gallstones should undergo cholecystectomy, although the management of asymptomatic gallstones is less clear. Laparoscopic cholecystectomy can be safely performed but associated common duct bile stones first require endoscopic retrograde cholangiopancreatography. As patients with SCD are living longer into adulthood, cirrhosis and liver failure are becoming increasingly prevalent disease-related sequelae. Sickle cell intrahepatic cholestasis syndrome (SCIC) is an uncommon but potentially fatal complication of SCD with a high death rate, observed mainly in patients with homozygous sickle cell anaemia. Its presentation ranges from a benign hyperbilirubinaemia to a fulminant hepatic failure and is characterised by abdominal pain in the upper right quadrant, enlarged liver, acholic or light stools, marked hyperbilirubinaemia and variable elevation of aminotransferases (AST and ALT) levels. In severe cases, renal dysfunction and alteration of coagulation tests can be observed. The diagnosis requires exclusion of viral hepatitis and cholecystitis/cholelithiasis. Treatment patterns are not well established and range from supportive care to exchange transfusion. Early recognition of severe cases and aggressive exchange transfusion is crucial for these patients. Vigilance for hepatic dysfunction, aggressive iron chelation in transfused patients and prompt recognition and treatment of hepatobiliary disease in children may prevent development of hepatic end-organ dysfunction in adults. Chelation therapy can also be associated with liver and kidney dysfunction that needs close monitoring.

Pregnancy

People with SCD are at risk for having a child affected with SCD if their partners have SCD, β-thalassemia trait or are carriers of other abnormal haemoglobin such as HbC. Women whose partners carry one of these traits can avoid an affected pregnancy by undergoing preimplantation genetic diagnosis (PGD). PGD is testing performed on an embryo during an *in vitro* fertilisation cycle. Alternatively, after spontaneous conception, prenatal diagnosis of SCD is possible by chorionic villus sampling in the first trimester or by amniocentesis in the second trimester of gestation.

Women with SCD are frequently exposed to blood products. The fetuses of women who are alloimmunised are at risk of significant haemolytic anaemia or mortality. The fetuses are at an increased risk of growth restriction, preterm delivery and stillbirth amongst women with SCD. Fetal surveillance, which includes growth ultrasounds and antepartum testing (nonstress tests, biophysical profiles and contraction stress tests), may lead to planned early delivery and can reduce but not eliminate risks. For women who require chronic opioid therapy during pregnancy, there is an increased risk of neonatal withdrawal in their newborns.

The steady-state haemoglobin level falls in SCD during pregnancy, similar to the decline in haemoglobin observed in normal pregnant women. Folate deficiency can exacerbate the anaemia and supplements should be provided throughout pregnancy. Compared to women without SCD, women with SCD are more likely to experience preeclampsia, venous thromboembolism, infections and maternal mortality during pregnancy. During pregnancy, 40–50% of women with SCD require at least one hospital admission. The presence of pulmonary hypertension increases the cardiopulmonary demands of gestation. Painful episodes become more common in the last trimester. Prophylactic transfusions during pregnancy or the type of delivery do not alter the outcome for mother or newborn however, prophylactic transfusions at regular intervals at the onset of pregnancy should be considered for women with a history of severe SCD-related complications to reduce recurrent pain episodes, acute chest syndrome or if the patient has a high-risk pregnancy from additional comorbidities such as other medical conditions or nephropathy.

In women with SCD, regular use of contraception can decrease the health risks associated with an unintended pregnancy. Hormonal contraceptives may also decrease menstrual blood flow, leading to higher haemoglobin levels. Use of progestin-only hormonal contraceptives lowers the risk of thromboembolism compared to use of oestrogen-containing contraceptives and has been shown to be safe for women with SCD. Intrauterine devices (IUDs) and intrauterine implants carry modest risks associated with the insertion procedure. Progestin-only contraceptives (pills, injections and implants), levonorgestrel IUDs, and barrier methods should be used as first-line contraceptive methods in women with SCD. If the benefits are considered to outweigh the risks, combined hormonal contraceptives (pills, patches and rings) may be used in women with SCD as second-line therapy.

Renal complications

There is increasing recognition that renal disease affects morbidity and mortality in individuals with SCD age. The hypoxic, acidotic and hypertonic renal medulla favours vaso-occlusion, leading to destruction of the *vasa recta* and hyposthenuria in the first year of life. It presents clinically as enuresis or nocturia, and patients are susceptible to dehydration in hot weather. Haematuria as a result of papillary necrosis usually originates from the left kidney. Management is generally by bed rest and hydration, although sometimes blood transfusion and ε-aminocaproic acid are required. SCD patients tend to have a lower systemic BP than age and ethnic-matched controls. Therefore, it is important to be aware of relatively 'normal' BP in patients whose baseline BP readings are typically low – this may indicate significant hypertension. Blood pressure goal for SCD patients should be ≤120–130/80 mmHg.

Proteinuria due to glomerular injury precedes development of nephrotic syndrome and eventual chronic renal insufficiency in one-quarter of adults. Individuals with SCD can develop proximal and distal renal tubular acidosis (RTA) that is characterised by low carbon dioxide (CO_2) and abnormal potassium levels, and occasionally hyperchloraemia. In addition, iron-chelating agents may cause Fanconi syndrome (generalised proximal tubular dysfunction), which may affect calcium and phosphate levels in the blood and urine. All patients should have annual screening starting at age 8 years for proteinuria with urinalysis and urine albumin-to-creatinine ratio and should have correction of their acidosis with bicarbonate as well as appropriate treatment for the RTA. The progression to renal failure can be delayed by angiotensin-converting enzyme inhibitors. Careful control of blood pressure, avoidance of non-steroidal anti-inflammatory drugs (NSAIDs) and aggressive treatment of urinary tract infection and anaemia are important objectives for patients with chronic renal insufficiency. Patients with end-stage renal disease are treated with dialysis and renal transplantation. Some renal complications, such as hyposthenuria and haematuria, are also observed in individuals with sickle trait, as is the rare renal medullary carcinoma.

Priapism

Priapism occurs in two-thirds of males with SCD, with a peak incidence in the second and third decades. Males do not reveal this often during regular history so early counselling is important during regular visits. It is caused by vaso-occlusion leading to obstruction of venous drainage from the penis. It typically affects the *corpora cavernosa* alone, resulting in a hard penis with a soft glans. Episodes can be brief (stuttering) or prolonged, when they last for longer than 3 hours. Recurrent priapism leads to fibrosis and eventual impotence. Young boys require explanation of symptoms and the need to seek early help for priapism. At the onset of priapism, patients should drink extra fluids and attempt to urinate. An oral dose of pseudoephedrine or terbutaline can be given. Persistent priapism requires intravenous hydration and opioid analgesia. If priapism persists for more than 2–3 hours, aspiration and irrigation of the corpora with dilute phenylephrine or etilefrine solution should be performed.

A simple intracavernosal injection with these agents tried early may induce detumescence and avoid the need for aspiration and irrigation. For patients with recurrent episodes, daily sildenafil or daily alpha-adrenergic agonist may be used. Antiandrogens, 5α-reductase inhibitors and gonadotropin-releasing hormone agonists with or without intermittent self-administration of sympathomimetic agents for acute episodes have been shown to be successful medical management options for some patients with stuttering priapism. As anti-androgenic agents, these therapies can negatively affect sexual function and physical composition. Therefore, they are not recommended in young patients who have not reached puberty or adult stature, and they should not be used in men desiring fertility. Priapism lasting for more than 4–6 hours is a urologic emergency because it represents a compartment syndrome of the penis. In these cases, urology should consider aspiration of blood from the corpus cavernosum with or without saline irrigation followed by injection of an alpha-adrenergic agonist to cause detumescence and in turn allow oxygenated blood to re-enter the cavernosa. For patients with recurrent episodes despite being on hydroxycarbamide, chronic transfusion therapy with a goal HbS <50% should be started.

Ocular complications

Vaso-occlusion of retinal and other vascular beds in the eye can lead to grave complications. Patients with SCD can develop abnormal (comma-shaped) conjunctival vessels, iris atrophy, retinal pigmentary changes and retinal haemorrhages. However, much more serious is neovascularisation causing proliferative retinopathy, appearing as a 'sea fan', with its potential for vitreous haemorrhage and retinal detachment. Such patients are treated with laser photocoagulation or vitrectomy. The incidence of proliferative changes is substantially higher in HbSC and HbS/β^+ thalassaemia patients than in HbSS. All patients with SCD should have annual ophthalmological evaluation, beginning in the second decade.

Sudden change in vision in a patient with SCD is an ocular emergency. Central retinal artery occlusion requires immediate treatment with hyperoxygenation and reduction of intraocular pressure, but the prognosis for vision is poor. Hyphaema, which can arise after minor trauma, leads to glaucoma due to sickling of blood in the anterior chamber. The elevated intraocular pressure causes ischemic optic atrophy and retinal artery occlusion. Individuals with sickle trait are also vulnerable to this complication. Urgent surgical attention is required to wash out blood from the anterior chamber.

Bone complications

The chronic haemolytic process results in expansion of the medullary space. Bone infarction due to vaso-occlusion produces tenderness, warmth and swelling, which can be difficult to distinguish from osteomyelitis. In such cases, cultures from blood and direct aspiration of the joint involved are negative and radiography later shows patchy sclerosis and cortical thickening. Collapse of vertebral end plates due to infarction produces the codfish appearance. Patients are managed with analgesia and hydration until resolution of symptoms.

Avascular necrosis of the femoral head is a serious complication that is difficult to treat and leads to chronic disability and pain (Figure 15.4d). Patients with coexisting α-thalassaemia have a higher incidence of osteonecrosis at a younger age. The condition also affects the humeral head but with fewer functional consequences. Aggressive physical therapy or combination of early core decompression followed by physical therapy can postpone the need for additional surgical intervention. Hip arthroplasty may be required for patients with severe symptoms.

The majority of children ages 9–19 years of age with severe manifestations of SCD have significant reduction in their bone density. Deficits in calcium and vitamin D occur in a majority of patients, which might contribute to low bone density, therefore vitamin D should be optimised in these patients.

Leg ulcers

Chronic leg ulcers are frequent in adult patients with SCD, particularly affecting males with the HbSS genotype (Figure 15.4e). Ulcers arise near the medial or lateral malleolus and may be single or multiple. Occlusion of skin microvasculature from sickle red cells predisposes to ulcers, which are made worse by trauma, infection or warm climate. Ulcers are always colonised with pathogenic bacteria (*Pseudomonas aeruginosa*, *S. aureus* and *Streptococcus* spp.) and acute infection can occur. The ulcers are painful and resistant to healing, and although bed rest and elevation of the leg are efficacious, they may not be practical, owing to the chronic nature of the problem. Treatment requires debridement, elastic dressings, zinc sulfate, and in some cases, red cell transfusions and skin grafting. Topical lidocaine is used for local treatment as with any leg ulcer in addition of NSAIDs and opioid therapy. Oedema related to superficial or deep venous insufficiency is usually seen. Venous compression is therefore recommended to relieve pain, reduce oedema and promote wound healing. Nitric oxide plays an essential role in chronic wound healing by increasing the production of extracellular matrix and stimulating keratinocyte proliferation and angiogenesis. A phase I study in 2014 on topical sodium nitrite, which produces NO, showed a significant reduction in the surface area of leg ulcers and in SCD patients. A recent study using the disease modifying agent voxelotor in patients with SCD and leg ulcers showed some improvement or resolution of the ulcers in the majority of patients on voxelotor therapy compared to patients on placebo. The resolution of ulcers was associated with increased haemoglobin levels in these patients.

Variant sickle cell syndromes

Sickle cell trait

Sickle cell trait (HbAS) is a benign condition that has no haematological manifestations and is associated with normal growth and life expectancy. The ratio of HbA to HbS is 60:40, owing to the greater affinity of α-globin chains for β^A-globin chains. Sickle cell trait affects 8–10% of African–Americans and up to 25–30% of the population in West Africa. Sickle trait reduces the risk of severe *falciparum* malaria, but not the prevalence of parasitaemia. There appears to be no effect on infections with other forms of malaria. Impaired urine-concentrating ability and haematuria can occur and an increased incidence of urinary tract infection is observed in pregnant women with sickle cell trait. Splenic infarction is possible at very high altitudes. A slight risk of sudden death during exercise has been reported. Sickling and vaso-occlusion under extreme circumstances can lead to rhabdomyolysis, acute kidney injury and cardiac arrhythmias. Like all people, individuals with sickle cell trait are advised to gradually increase exercise intensity, to avoid dehydration and to stop physical activity with the onset of muscle cramp or fatigue. Recent data shows some concerns for higher risk of venous thromboembolism, specifically pulmonary emboli, in people with sickle cell trait compared to non-sickle cell trait patient but further data is needed to clarify this risk. Treatment of sudden collapse consists of rapid intravenous hydration and oxygen supplementation.

Genetic counselling should be provided to individuals with sickle cell trait. It is important to note that some patients with HbS/β^+ may be misdiagnosed as having sickle cell trait. Therefore, it is important to look at the haemoglobin electrophoresis/high-performance liquid chromatography of patients to confirm the diagnosis. A rare compound heterozygote for haemoglobin S and haemoglobin Quebec-Chori may present as sickle cell trait on electrophoresis but have clinical and laboratory evidence of mild-to-moderately-severe SCD. Therefore, careful examination of the peripheral blood film and of a complete blood count, including a reticulocyte count, is needed to establish a diagnosis of sickle cell trait. When sickle forms are seen in the peripheral blood smear, the patient has evidence of haemolytic anaemia, and when atypical symptoms suggestive of SCD occur in a patient with an apparent sickle cell trait, an evaluation for an electrophoretically silent haemoglobin variant is indicated.

HbSC disease

HbC is found amongst individuals of African descent and the compound heterozygous state HbSC accounts for 25–50% of patients with SCD. The vaso-occlusive complications seen in patients with HbSC resemble those seen in patients with HbSS but are less severe. Splenomegaly and the risk of sequestration can persist into adult life. Of particular note is the higher incidence of proliferative retinopathy in HbSC beginning in the second decade. The haemoglobin level (100–120 g/L) is higher than in HbSS, and the red cells are relatively microcytic with a higher mean corpuscular haemoglobin concentration (MCHC). Peripheral blood smear reveals frequent target cells, intraerythrocytic crystals and rare sickle cells. Equal amounts of HbS and HbC are present in the red cells and the solubility test for sickle haemoglobin is positive. The electrophoretic appearance of HbSC, HbSE and HbSO Arab at pH 8.4 is similar, but a distinction can be made based on ethnicity and by performing isoelectric focusing or agar gel electrophoresis at pH 6.5. Fat embolism syndrome (FES) is a rare clinical entity that results from the release of fat globules into the circulation, causing respiratory and neurologic damage, cutaneous and haematological manifestations and can occur in individuals with HbSC disease. People with haemoglobin C trait have red blood cells and do not have health problems related to having the trait. However, they can pass haemoglobin C trait to their children and combined with HbS trait can have a child with HbSC disease.

Sickle cell/β-thalassaemia

Sickle cell/β-thalassaemia compound heterozygotes account for less than 10% of patients with sickle syndromes in the US. The majority of these patients have the β^+-phenotype, with the proportion of HbA ranging from 3 to 25%. The clinical phenotype is mild and disease severity correlates with the amount of HbA present. The clinical manifestations of the less frequent HbS/β^0-genotype are similar in severity to those of HbSS. The red cells are microcytic and hypochromic, and variable numbers of target cells and sickle cells are observed. Reticulocytosis (10–20%) is present and the level of HbA_2 is elevated.

Sickle cell anaemia with coexistent α-thalassaemia

Coinheritance of αα-thalassaemia (−α/αα or −α/−α) with SCD is common, and such patients have less severe anaemia and demonstrate hypochromia and microcytosis. In general, the clinical severity is similar to that seen in HbSS patients with a normal complement of α-globin genes.

Sickle cell/HPFH

Approximately 1 in 100 patients with HbSS has an elevated HbF level due to deletional or non-deletional mutations that maintain γ-globin gene expression after birth. Such individuals have 20–30% HbF and less than 2.5% HbA2. In most newborn screening techniques, HbS/HPFH appears identical to severe HbSS disease. Therefore, DNA globin gene testing or family studies looking at both parents' sickle cell trait is necessary to make this diagnosis. The haemoglobin level is normal, with microcytosis,

and target cells are observed in the peripheral smear. The clinical course is benign, and vaso-occlusive complications are rare because of the inhibition of sickling by elevated HbF.

Other sickling syndromes

Sickle cell/Hb Lepore disease

Coinheritance of Hb Lepore with the sickle cell mutation produces a clinical picture similar to that of HbS/β-thalassaemia, but with a low HbA_2 level.

Sickle cell/HbD disease

Of all the D or G haemoglobins, HbD Punjab (D Los Angeles) alone interacts with HbS to produce moderately severe haemolytic anaemia in compound heterozygotes. Target cells and irreversibly sickled cells are observed in the peripheral smear, and the clinical manifestations resemble mild sickle cell anaemia.

Sickle cell/HbO Arab disease

HbO Arab resembles HbC on alkaline electrophoresis and produces a moderately severe haemolytic anaemia in association with HbS. The disease is more severe than HbSC, and numerous sickled erythrocytes are observed on the peripheral smear.

Sickle cell/HbE disease

HbSE disease causes mild haemolysis and no remarkable abnormality of red blood cell morphology. HbE comprises only 30% of the total haemoglobin because of the thalassaemic nature of the mutation. Patients are generally asymptomatic, although occasionally significant vaso-occlusive complications and anaemia have been observed.

Diagnosis

Peripheral blood findings

The peripheral blood picture depends on the type of sickle cell syndrome. The haemoglobin level is normal in the newborn period, but anaemia develops and sickle or cigar-shaped irreversibly sickled cells can be observed in the peripheral blood by 3–4 months of age as HbF declines. In HbSS disease, the red cells are normocytic and normochromic, with polychromasia, many irreversibly sickled cells (ISCs) and fewer target cells (Figure 15.5a). The average reticulocyte count is 10% (4–20%) and normoblasts may be observed. Red cells are microcytic in the presence of coexisting α-thalassaemia or iron deficiency. In HbS/β-thalassaemia, ISCs, target cells and hypochromic microcytic red cells are prominent. The red cell morphology in HbSC disease is characterised by predominant target cells and rare ISCs. The occasional Howell–Jolly body, indicative of loss of splenic function in SCD, may be observed. The white cell count is elevated ($12–20 \times 10^9$/L) as a result of an increase in mature neutrophils. The platelet count is also elevated to $300–500 \times 10^9$/L as a result of decreased splenic function.

Other laboratory tests

Serum levels of unconjugated bilirubin, reticulocyte counts and lactate dehydrogenase (LDH) are elevated, and haptoglobin and haemoglobin levels are decreased in patients with haemolysis related to SCD. SCD alters other organ functions including the coagulation system, renal and cardiac functions.

Haemoglobin electrophoresis

HbS can be identified by cellulose acetate electrophoresis at pH 8.4 (Table 15.1 and Figure 15.5b). HbD and HbG have the same electrophoretic mobility with this method, but can be distinguished using citrate agar electrophoresis at pH 6.2 or thin-layer isoelectric focusing. Distinction cannot be made between HbSS and HbS/β^0 -thalassaemia on electrophoresis. The diagnosis of HbS/β^0 -thalassaemia is suggested by microcytosis and elevated HbA_2, and confirmed by finding β-thalassaemia trait in one of the parents. HbA and HbS are observed upon electrophoresis in both sickle cell trait and HbS/β^+ -thalassaemia; however, the HbA fraction is greater than 50% in the former, but ranges from 5 to 30% in the latter. The level of HbF is variably elevated, with higher levels observed in patients with the Arab–Indian and Senegal haplotypes.

High performance haemoglobin liquid chromatography, or capillary electrophoresis, is widely used in lieu of gel electrophoresis and provides qualitative and quantitative estimations of haemoglobin fractions in an automated fashion. Globin gene testing is being increasingly used for the diagnosis of SCD given its increased specificity and decreasing costs.

Other tests to detect sickle haemoglobin

Sickling of red cells can be induced by sealing a drop of blood under a coverslip to exclude oxygen or by adding 2% sodium metabisulfite. The solubility test for HbS utilises a reducing agent such as sodium dithionite, which is added to the haemolysate. Deoxy-HbS is insoluble and renders the solution turbid. Both these tests are unable to distinguish sickle cell trait from sickle cell anaemia and cannot be used for primary diagnosis. They are useful aids in the identification of an abnormal electrophoretic band as HbS and for identifying sickle cell trait in units of red cells prior to transfusion.

Newborn screening

Universal newborn screening is recommended for identifying SCD in the neonatal period. The efficacy of penicillin prophylaxis in preventing death from early sepsis in SCD provided the rationale for development of screening programs. Blood samples

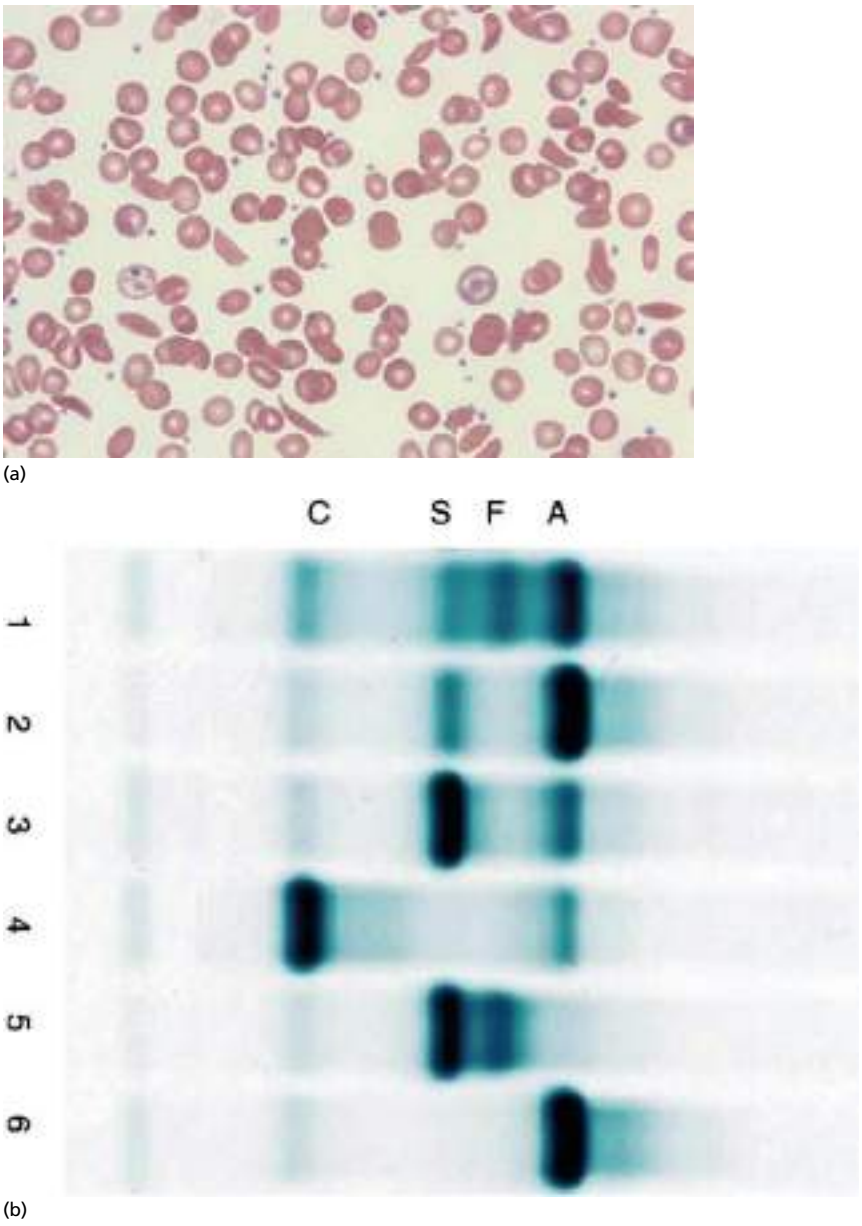


Figure 15.5 (a) Peripheral smear from an individual with sickle cell anaemia (Source: American Society for Clinical Pathology / https://www.ascp.org/patient-champion/resources/sickle-cell-disease / last accessed on November 22, 2024). (b) Haemoglobin Electrophoresis showing Lane 1 as control; Lane 6 as normal adult HbAA; Lane 5 as an infant with HbSS and residual HbF production; Lane 4 as HbCC disease; Lane 3 as HbSS prior to transfusion; and Lane 2 is the same patient after transfusion (Source: Menon & Frei-Jones (2021) / Springer Nature).

obtained by heel prick are spotted onto filter paper and tested by electrophoresis or chromatography. Neonates with HbSS disease and HbS/β^0-thalassaemia have an FS pattern (the order of haemoglobins indicates their relative abundance in the sample). In sickle cell trait, the haemoglobin pattern is FAS, whereas newborns with HbS/β^+-thalassaemia have an FSA pattern. Finally, the presence of the FSC pattern suggests HbSC disease. Family studies help to make the definitive diagnosis and, when both parents are unavailable, DNA-based testing is useful.

Prenatal diagnosis

Prenatal diagnosis is available through direct detection of the GAG→GTG mutation responsible for SCD in fetal cells. Chorionic villus sampling is recommended given its reliability in making an early prenatal diagnosis with minimal risks. Preimplantation diagnosis and selection of healthy embryos may offer some hope in minimising disease burden in the future. All patients with abnormal newborn screening results

should receive genetic counselling in addition to follow-up with a provider to assess their clinical and laboratory outcomes and severity of disease that is not evident on newborn testing alone.

Therapy

This section discusses general issues in the management of SCD. The treatment of specific complications is addressed in the section on clinical manifestations.

Routine healthcare

All adults and children with SCD require specialised care at an experienced centre with expertise in taking care of these patients.

The level of healthcare available to patients with SCD varies tremendously in different countries. Where resources are limited, the primary focus should be on penicillin prophylaxis, vaccination, education and analgesia for painful episodes. Recent studies have shown that hydroxycarbamide and TCD screening for stroke can be successfully implemented in low-resource settings with success. Where comprehensive care is available, both medical and psychosocial needs should receive attention (Figure 15.6). Sickle cell centres should have specialists in several fields who are available to address complications that may affect different organs.

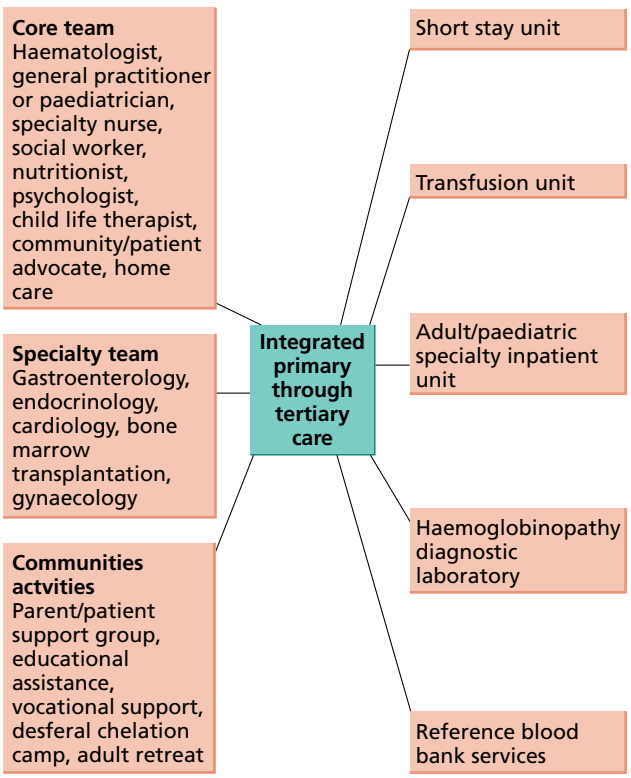


Figure 15.6 Comprehensive care of patients with sickle cell disease.

In cases when diagnosis is made on newborn screening, the infant should be seen within 1–2 months to instruct parents about infections and splenic enlargement. Routine immunisation should include pneumococcal, *H. influenzae*, hepatitis B and influenza vaccines. All children receive prophylactic penicillin; evidence for its continuation after the age of 5 years in the absence of any episode of pneumococcal sepsis or splenectomy is unclear and practices vary. Folic acid supplementation (1 mg daily) is recommended. Evaluation of cerebral blood flow by transcranial Doppler should be performed on all children after 2 years to identify those at risk for stroke. Annual retinal examination is begun at 10 years. Sexually active women should have routine pelvic examinations and receive advice about birth control.

Infections

Fever in children with SCD requires urgent attention in the clinic or emergency department. A complete blood count, blood and urine cultures and chest radiographs should be obtained, and lumbar puncture should be performed if meningitis is suspected. Very young children with fever or older children with apparent sepsis should be hospitalised for intravenous antibiotics. The choice of antibiotics depends on causative agents prevalent locally and the pattern of resistance. In the USA, cefuroxime or ceftriaxone are preferred, whereas high-dose penicillin is used in several other countries. Many patients older than 2 years who do not look septic or seriously ill can be managed at home after receiving parenteral antibiotics in the emergency department. Antibiotics should continue for 1 week when bacteraemia is documented. In the presence of pneumonia, a macrolide should be added to cover *Mycoplasma* or *Chlamydia*. Antibiotics for osteomyelitis should provide coverage for *Salmonella* and *S. aureus* and are given for a period of 4–6 weeks.

Transfusion therapy

Blood transfusion in SCD is used to treat severe anaemia or to reduce the amount of circulating sickle haemoglobin. Only sickle-negative blood, which can be identified by negative sickle solubility test, is used for transfusions. The blood should also be leuko-depleted and matched for common minor E, C and Kell antigens. A simple transfusion is used to treat severe anaemia that is often associated with aplastic crisis and splenic sequestration. Older patients with chronic kidney injury may also need transfusions for declining haemoglobin level.

Dilution of circulating sickle haemoglobin can be accomplished by simple transfusion if the baseline haemoglobin level is low. Exchange transfusion is required to prevent hyperviscosity from the significant rise in haemoglobin when the patient has high baseline haemoglobin or when a greater reduction in

HbS is desired. The final haemoglobin level should not exceed 120 g/L after simple or exchange transfusion. Conditions in which a reduction in the proportion of HbS is required include stroke, progressive acute chest syndrome, persistent priapism or preparation for general anaesthesia longer than 1 hour. Longer-term reduction in HbS through regular transfusions is advocated to prevent recurrence of stroke and sequestration, and in selected patients with leg ulcers or chronic pain. Routine blood transfusion is not needed for pain episodes, infections, minor surgery or uncomplicated pregnancy.

It is possible to eliminate most complications of SCD with the use of chronic transfusions to suppress endogenous sickle haemoglobin production. However, alloimmunisation, iron overload and transmission of viruses are significant risks that limit the use of transfusions for the management of severe complications. In addition, because of the limited availability and decreased safety of blood, criteria for transfusion are more stringent in less developed countries. The high incidence of alloimmunisation from minor blood group incompatibility (Rh, Kell, Duffy and Kidd) between donors and recipients can be avoided by use of phenotypically matched units. Genomic analysis of RH alleles can improve the precision of blood matching and decrease the incidence of alloimmunisation. Patients on long-term transfusions develop iron overload, which requires chelation with deferoxamine, deferasirox or deferiprone. The prevalence of iron-induced organ damage appears to be lower in SCD compared with thalassaemia, despite similar amounts of iron burden. MRI and, rarely, liver biopsy are necessary to measure iron burden because the serum ferritin is unreliable. Because iron accumulation can be reduced or prevented by erythrocytapheresis (red cell exchange), this technique is now preferred when venous access is available.

Pain management

Prompt management of pain is essential, given its frequent occurrence and potential adverse psychological consequences. Patients with recurrent pain are best managed in a familiar ambulatory setting rather than the emergency department (ED). When ED management is necessary, utilization of pain management protocols can improve the delivery of care. Pain management protocols have been shown to expedite the administration of pain medications and facilitate timely reassessment of pain withing 30 minutes of triage in the ED. The patient should be evaluated for potential infectious, traumatic or surgical causes of pain. Pain assessment tools are available for young patients and are also helpful in older patients to follow the response to therapy. Adequate analgesia using opioids and NSAIDs should be provided. Several narcotic agents are available for oral and parenteral use and the choice of medicine depends on local experience, as well as the patient's preference. Even though no opioid alternatives are available, ketamine has shown some promise in its effects on pain and narcotic-sparing effects. The use of incentive spirometry reduces the potential for developing hypoxia and acute chest syndrome secondary to hypoventilation. Under-treatment of pain can be avoided by using patient-controlled analgesia, which has the added benefit of reducing apparent drug-seeking behaviour. Narcotic addiction is no more frequent in sickle cell patients than in others requiring analgesia. NSAIDs improve pain control with or without narcotics. Providing psychosocial support and reassurance, and allaying anxiety, are important goals. Individualised pain plans should be developed for all patients with SCD with the participation of the patient and their family and should be available to be used in acute settings when patients present with an acute pain event. Chronic pain is rare in SCD and may require long-acting pain management with the support of a pain specialist. It is important to note that overuse of NSAIDs can lead to renal dysfunction in SCD patients at risk for renal complications and should be monitored.

Disease-modifying therapies

Hydroxycarbamide

Hydroxycarbamide is a tremendously important drug in the management of patients with SCD who have severe clinical manifestations. Hydroxycarbamide inhibits ribonucleotide reductase, leading to S-phase arrest of replicating cells, and is used in SCD because of its ability to stimulate production of HbF. Hydroxycarbamide increases HbF as a result of stress erythropoiesis induced by its myelosuppressive effect. Patients show variable response in the degree of rise in HbF, and some experience no change from the baseline value. Other biological effects of hydroxycarbamide play an equally significant role in the beneficial clinical effects observed during therapy. Erythrocytes of patients on hydroxycarbamide have increased water content and deformability and decreased adherence to vascular endothelium. There is elevation of the haemoglobin level, mean corpuscular volume (MCV), HbF and F cells, whereas total white cell and neutrophil count, reticulocyte count and the number of dense sickle cells decrease. Patients on hydroxycarbamide experience 50% reduction in the incidence of acute painful episodes and acute chest syndrome. Transfusion needs, frequency of hospital admissions and the risk of death are also decreased. Hydroxycarbamide does not improve leg ulcers, while the effect on priapism is unclear. It may reduce the risk of stroke, and its role in children with elevated cerebral blood flow velocities is under intense study.

The efficacy of hydroxycarbamide therapy is now well established in adults and children as young as 9 months of age. Prospective trials in paediatrics demonstrated a reduction in both painful episodes and acute chest syndrome.

The overall benefit of hydroxycarbamide to the patient is closely related to compliance, for which monitoring in the clinic and psychological support should be provided. Patients

should be made aware that 3–6 months may elapse before clinical benefits are realised. Hydroxycarbamide should be offered to all patients ages 9 months and above with HbSS and HbSβ0 disease regardless of their incidence of pain crises and acute chest syndrome episodes, and symptomatic patients with other genotypes of sickle cell anaemia. It is started at a dose of 20 mg/kg per day and increased by 5 mg/kg every 2–3 months until the absolute neutrophil count is close to 2.5×10^9/L. Compliance with prescribed dose should be ascertained before each dose escalation, up to the maximum dose of 35 mg/kg or 2000 mg/day. Patients require frequent monitoring of blood counts, as well as renal and hepatic function. Myelosuppression is the most commonly encountered side-effect and temporary cessation of therapy and dose reduction is required for neutropenia, thrombocytopenia, reticulocytopenia or fall in haemoglobin. Dose modification is necessary for patients with renal dysfunction. Skin pigmentation affecting the nails, palms and soles is commonly observed. There may be a temporary decline in sperm production amongst men while on hydroxycarbamide treatment. Despite concerns about the leukaemogenic and teratogenic effects of hydroxycarbamide, no convincing increase has been reported in SCD. Men and women should practise contraception while taking hydroxycarbamide, and women who become pregnant should stop the drug. No decrease in growth rate is observed in children over 5 years using hydroxycarbamide, while the growth effects of the drug in younger children are under evaluation. Overall, the risks associated with hydroxycarbamide therapy are low. A recent study in children with sickle cell anaemia in sub-Saharan Africa treated with hydroxycarbamide showed its feasibility and safety in this population and reduced incidence of vaso-occlusive events, infections, malaria, transfusions and death. This study supports the need for wider access to treatment for sickle cell anaemia.

L-Glutamine (Endari)

L-Glutamine may be used for SCD for patients with recurrent pain episodes despite being on hydroxycarbamide or who could not tolerate hydroxycarbamide therapy. It contributes to NAD+ synthesis for the regeneration of glutathione, which is depleted when reactive oxygen species are circulating. It has been shown to reduce the number of annual vaso-occlusive episodes, hospitalisations and acute chest syndrome when used with hydroxycarbamide therapy. This therapy is currently approved for patients ages 5 years and older with HbSS and HbSβ0 with symptoms not adequately controlled with hydroxycarbamide alone.

Voxelotor

Voxelotor is a first-in-class small molecule that forms a reversible covalent bond with alpha-globin which stabilises dexoxygenated haemoglobin in the relaxed state, thus preventing polymerisation. This medication may be used for patients aged 12 years and over and has been shown to increase the haemoglobin lifespan and reduce haemolysis. No long-term safety or efficacy outcomes have been measured yet for this drug. However, long-standing anaemia is a known risk factor for many chronic complications of SCD including pulmonary hypertension, chronic kidney disease, cerebrovascular accidents and mortality, so correction of anaemia with voxelotor has potential to preserve long-term organ function. This therapy can be used in conjunction with hydroxycarbamide by mouth and is especially beneficial for patients who have low baseline haemoglobins.

Crizanlizumab

P-selectin is an adhesion molecule present on endothelial cells and platelets that facilitates interactions between endothelial cells, WBC, RBCs and platelets. P-selectin is known to be upregulated in inflammatory conditions including SCD. Crizanlizumab binds to P-selectin glycoprotein ligand and blocks adhesion and has been shown to decrease the incidence of vaso-occlusive pain episodes, longer time to first and second crises. It has not been shown to decrease any markers for haemolysis. This therapy may be used for patients with all genotypes of SCD aged 16 years and over. It is delivered intravenously every 4 weeks.

In young patients with severe disease, matched-sibling stem cell transplantation should be discussed as an alternative to chronic transfusions or long-term disease-modifying therapies listed above.

New therapeutic modalities

A better appreciation of the pathophysiology of SCD will make it possible to exploit new therapeutic mechanisms (Table 15.4). There are several agents in clinical trials that are promising.

Haemopoietic stem cell transplantation

Allogeneic haemopoietic stem cell transplantation (HSCT) from a matched sibling donor cures 93% of children with SCD less than 16 years of age and 81% of patients over 16 years or older. The 5-year probability of graft versus host disease-free survival is 86% for patients younger than 16 years and 77% for patients 16 years or older. Both bone marrow and umbilical cord blood from related donors are suitable sources for the stem cells (Figure 15.7). Studies are underway to evaluate the use of unrelated donors or reduced-intensity conditioning regimens. Additional long-term risks after HSCT are infertility and second malignancy. Selection of candidates for HSCT is complex owing to the uncertain long-term course of the disease. HSCT should be considered in children (age <16 years) with SCD (any genotype) who have a human leucocyte antigen (HLA)-identical related donor and evidence of target organ damage involving

Table 15.4 Summary of disease-modifying therapies currently available.

Drugs	Approved for ages	Mechanism of action	Major side effects	Administration	Good for..
Hydroxycarbamide	>9 months	Ribonucleotide reductase inhibitor: ↑HbF, ↓WBC, retic	Cytopenia	Oral liquid or capsule	All patients with HbSS or HbSβ0 or other genotypes with severe phenotype
L-glutamine	≥ 5 years	Repletes glutathione to reduce oxidative stress	Nausea, abdominal discomfort	Oral powder for reconstitution	Persistent pain on HU
Voxelotor	≥12 years	Stabilises HbS in deoxygenated state and reduces polymerisation	Headache, abdominal discomfort	Oral tablet	Low Hb, HU not effective or tolerated
Crizanlizumab	≥16 years	Binds to PSGL-1 and prevents P-selectin mediated adhesion of WBC, sickled RBCs and platelets to endothelium and each other	Arthralgias, Nausea	IV infusion	Inflammatory phenotype, frequent pain crises, HU not effective or tolerated

Table 15.5 Advances in the management of sickle cell disease.

Category	Intervention
Newborn screening	Counselling
	Comprehensive care
Infection	Prophylactic penicillin. Immunisation
Brain injury	Screening with TCD, MRI
	Neurocognitive testing, Behavioural health screening
	Chronic transfusions
Transfusion safety and iron overload	Phenotypically or genotypically matched red blood cells
	Erythrocytapheresis
	Iron chelation
Lung injury	Incentive spirometry
	Antibiotics (including macrolides)
	Transfusion
	Echocardiographic screening for pulmonary hypertension
	Prevention with hydroxycarbamide
Surgery/anaesthaesia	Preoperative transfusion for sedation >1 hour
Avascular necrosis	Physical therapy
	Hip joint replacement
Priapism	Adrenergic agonist
	Anti-androgen therapy
Pain	Prevention with hydroxycarbamide
	Patient-controlled analgesic devices
	Non-steroidal anti-inflammatory drugs
Renal	ACE inhibitors for proteinuria
	Improved renal transplantation
Gallbladder disease	Laparoscopic cholecystectomy
Severe disease	Allogeneic bone marrow transplantation
	Chronic transfusions
	Hydroxycarbamide, Voxelotor, Crizanlizumab, L-Glutamine

ACE, angiotensin-converting enzyme; MRI, magnetic resonance imaging; TCD, transcranial Doppler.

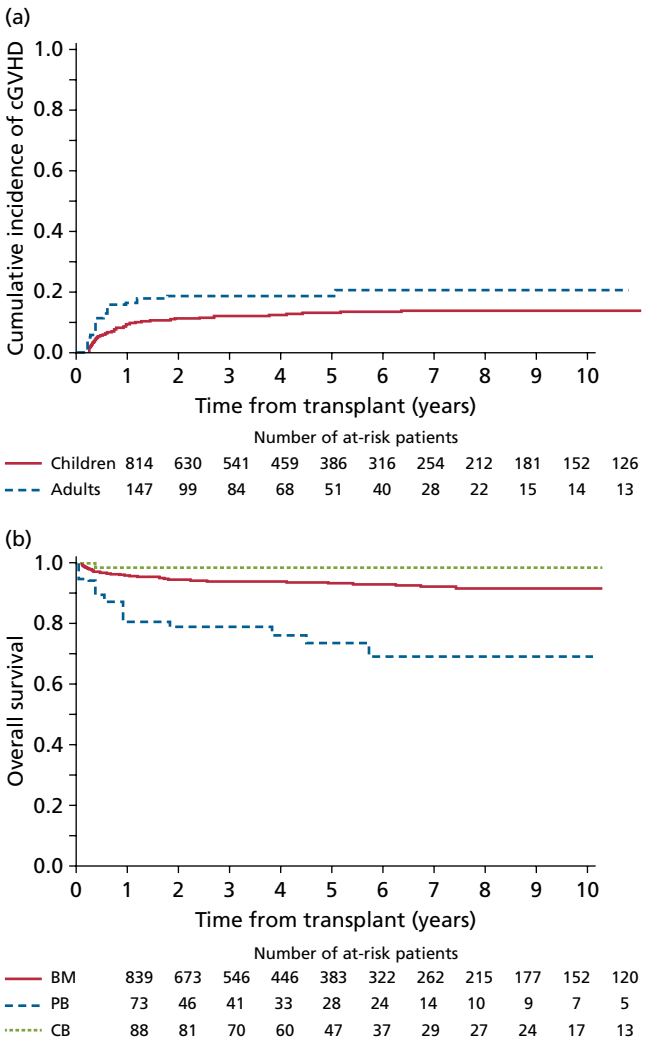


Figure 15.7 Outcomes after HSCT for SCD (a) Unadjusted chronic graft versus host disease, according to age; and (b) overall survival, according to stem cell (Source Gluckman E, *et al.* 2017/ with permission of Elsevier. *Blood.*)

the brain, lungs, kidneys or eyes. Children who are placed on long-term blood transfusions for any indication should also be evaluated for HSCT. Although it is clear that high-risk patients benefit from this treatment, the role of HSCT in asymptomatic children is not defined. Severe organ dysfunction increases the risks from the procedure, and hence discussion for HSCT should be started early when eligible indications are identified.

Gene therapy

Correction of SCD by gene therapy requires efficient insertion of a gene into repopulating haemopoietic cells and regulated expression in erythropoietic lineages. An anti-sickling haemoglobin, constituting 20–30% of the total haemoglobin, would be enough to produce clinical response. Mouse models of SCD have considerably helped in the effort to develop gene therapy and correction of the sickling phenotype has been demonstrated in such animals. Early ongoing clinical trials for gene therapy using CRISPR gene editing technique are very promising.

Psychosocial issues

Recurrent pain and the unpredictable course of the illness place SCD patients at higher risk of depression and anxiety with poor family relationships. Despite this, with integrated medical care and social support most patients with SCD are well-adjusted. Addiction to narcotics is an uncommon phenomenon and is the result of social influences rather than analgesic therapy. Attention to psychological wellbeing as well as educational and vocational support are important components of the care provided to SCD patients.

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CHAPTER 16

Hereditary disorders of the red cell membrane and disorders of red cell metabolism

16

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Haemolysis

Definitions

Haemolysis indicates that the destruction of red cells is accelerated. Normally, in adults, the bone marrow output is well below its maximal capacity. Red cell production can be increased more than 10-fold in the adult by increasing the cellularity of existing haemopoietic marrow, as well as by expansion of haemopoietic marrow into the long bones. In the newborn, and during infancy, marrow expansion depends on expanding the medullary cavity of bones, leading to thinning of cortical bone. These bony changes are most extreme in the β-thalassaemia syndromes, but some skeletal changes, usually some bossing of the frontal bones, may be seen in more extreme hereditary haemolytic anaemias of other causes. Increased red cell destruction is often completely matched by increased production, resulting in compensated haemolysis. When the rate of haemolysis exceeds the maximum erythropoietic capacity of the bone marrow, or when the latter is limited (e.g. because of inadequate supply of iron or folate or by ineffective erythropoiesis), the result is haemolytic anaemia.

General features of haemolysis

The clinical and laboratory aspects of haemolysis depend on the consequences of increased red cell destruction and production as well as the main process by which destruction takes place. Increased red cell destruction leads to an increase in unconjugated bilirubin from increased haemoglobin turnover. Unconjugated bilirubin does not appear in the urine, although there will be an increase in urinary urobilinogen. The bilirubin level is usually not more than 2–3 times normal because the normal liver is able to increase excretion to compensate for at least some of the increased production. Jaundice is usually mild in hereditary haemolytic anaemias although there are important exceptions.

In the neonate, particularly premature infants, liver function is not fully developed and more severe jaundice requiring urgent therapeutic intervention may occur. A rare but potentially confusing problem is the coinheritance of Gilbert syndrome, which comprises a group of congenital liver enzyme deficiencies that impair bilirubin conjugation. On its own, Gilbert syndrome does not produce clinical jaundice except when there is inadequate calorie intake, but in conjunction with haemolytic anaemia the hyperbilirubinaemia may be considerable. The increased bilirubin of haemolysis does increase the risk of gallstones and cholecystitis, which in turn may lead to an increase in serum bilirubin.

In the degradation of haemoglobin, the molecule is broken down into two constitutive subunits, α and β globin, which are bound to haptoglobin, the complex being rapidly internalised in the hepatocyte after binding to the haptoglobin complex receptor. In the presence of haemolysis, serum haptoglobin levels are

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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greatly reduced or absent. However, haptoglobin is an acute-phase protein and levels will increase in the presence of inflammation. Haemopexin is another haem-binding protein produced by the liver that is decreased in haemolysis. Chronic haemolytic anaemia may increase the iron content of the body through increased iron absorption as a consequence of anaemia coupled to the retention of the haem iron following binding to haptoglobin and haemopexin. In rare cases of inherited haemolytic anaemia, this iron overload may be sufficient to produce clinically important effects, particularly if there is coinheritance of a haemochromatosis gene. In most haemolytic anaemias, owing to membrane defects, the destruction of red cells takes place extravascularly in the reticuloendothelial system, particularly in the spleen, and the iron is retained. When destruction is intravascular, free haemoglobin will be released into the plasma, producing haemoglobinaemia and methaemalbuminaemia, and will pass through the glomerulus to produce haemoglobinuria and haemosiderinuria. Iron deficiency is thus more likely than overload in intravascular haemolysis.

Increased red cell production leads to expansion of the red cell precursor compartment of the bone marrow, as described above. There are also changes in the structure of the marrow as a consequence of the chronic anaemia, which allows the early release of reticulocytes and, in more marked cases of haemolytic anaemia, nucleated erythroblasts and even myelocytes. In the peripheral blood, the polychromasia and macrocytosis of reticulocytosis are the result of this increased throughput and release. The increased cell production requires an increased supply of folate which, at least theoretically, can produce folate deficiency unless supplements are given. It is usual to give folic acid (400 µg daily or 5 mg once weekly) to people with chronic haemolytic anaemia. The main features of haemolytic anaemia are summarised in Table 16.1.

Table 16.1 Main features of haemolytic anaemia.

Increased red cell destruction
Unconjugated hyperbilirubinaemia
Mild jaundice
Increased risk of gallstones
Increased urinary and faecal urobilinogen
Decreased serum haptoglobin and haemopexin
Extravascular changes
Increased iron stores
Splenomegaly
Intravascular changes
Haemoglobinaemia and haemoglobinuria
Haemosiderinuria
Methaemalbuminaemia
Decreased iron stores
Increased red cell production
Marrow expansion: bone changes
Increased erythropoiesis: ↓ myeloid/erythroid ratio
Reticulocytosis: polychromasia
Increased folate requirements: macrocytosis

Classification

Because of the unique structural and functional specialisation of the mature red cell, the impact on it of a wide range of exogenous or endogenous changes is relatively uniform: the cell will be destroyed prematurely. According to the site of the primary change, haemolytic disorders have been traditionally classified as being due either to intracorpuscular or to extracorpuscular causes. According to the nature of the primary change, haemolytic disorders have also been classified as inherited or acquired. These two classifications correlate almost completely with each other, in that extracorpuscular causes are usually acquired, whereas intracorpuscular causes are usually inherited. One notable exception is paroxysmal nocturnal haemoglobinuria, a disease in which an intracorpuscular defect is acquired as a result of a somatic mutation (see Chapter 31).

Although in every cell all molecules and organelles are naturally interdependent, it is convenient to consider the red cell as a conveyance for a large amount of haemoglobin contained in a plasma membrane, the stability of which is maintained by appropriate metabolic machinery and structural organisation of the proteins. Unfavourable genetic changes in any of these components may cause haemolysis. Accordingly, inherited haemolytic disorders can be classified into three major groups: (i) genetic disorders of haemoglobin (see Chapters 14 and 15); (ii) abnormal membrane (including the cytoskeleton) and (iii) abnormal metabolism (enzymopathies).

Red cell membrane disorders

The red cell membrane

The red cell membrane, like all other cell membranes, consists of a lipid bilayer that is stabilised and given specific properties by the proteins, glycolipids and other specialised molecules and structures with which it is associated. The lipid bilayer consists of approximately equal molar quantities of phospholipids and cholesterol molecules. The charged phosphatidyl groups of the phospholipids are hydrophilic and form the outer and inner surfaces of the bilayer. The interior of the membrane is formed by hydrophobic bonding of the acyl chains and cholesterol, which form the internal parts of the two leaflets (Figure 16.1). The arrangement is energy efficient but the two leaflets are not symmetrical. The outer leaflet consists mainly of phosphatidylcholine and sphingomyelin, the inner leaflet of phosphatidylethanolamine and phosphatidylserine (Figure 16.2). Maintenance of the asymmetry and the proper function of the membrane requires energy. In mature red cells, this is provided by adenosine triphosphate

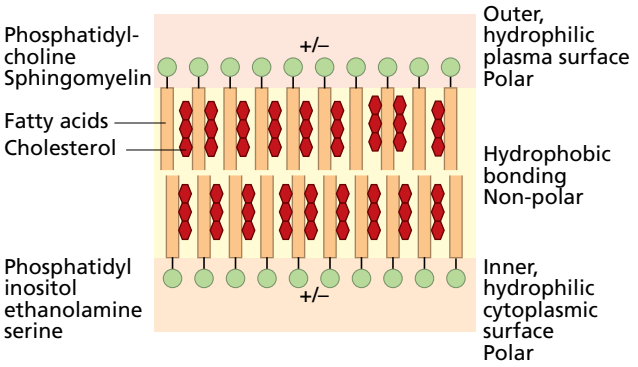


Figure 16.1 Arrangement of membrane lipids. The acyl chains of the diacylphosphatidylglycerides are hydrophobic non-polar domains and they form hydrophobic bonds with the acyl groups of the opposite layer. Cholesterol is present in roughly equimolar amounts and determines the fluidity of the membrane.

(ATP) from the glycolytic pathway and reducing power mainly in the form of glutathione.

The normal biconcave shape and function of the red cell membrane are determined by the membrane proteins and their interactions with the lipid bilayer and with each other. There are two main sorts of protein–membrane associations. The integral proteins have strong hydrophobic domains that are associated with the hydrophobic part of the bilayer. Many of these integral proteins span the membrane and provide channels between the plasma and cytosolic compartments. The cytosolic inner domains of these proteins interact with each other and with the second main group, the proteins of the membrane skeleton. The integral proteins that provide the

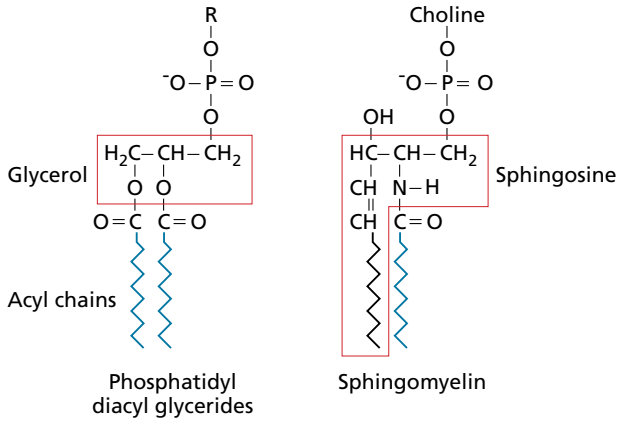


Figure 16.2 Main lipids of the red cell membrane. The outer, plasma, layer contains mostly neutral lipids, sphingomyelin and phosphatidylcholine (lecithin). The inner, cytoplasmic, layer contains mostly acidic groups, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol. R may be choline, serine, ethanolamine or inositol.

links between the lipid bilayer and the membrane skeleton have conveniently been referred to as 'vertical connections', whereas the proteins of the membrane skeleton that comprise the inner network of the cell membrane are characterised as 'horizontal connections'. Genetic abnormalities that produce spherocytosis mainly have mutations affecting the vertical connections. Mutations of the horizontal system usually produce elliptocytosis or more bizarre-shaped changes. The main proteins are listed in Table 16.2, and their arrangement is shown schematically in Figure 16.3.

In addition to the compartments mentioned so far, there are numerous surface proteins that provide the main interface with the plasma, including the blood group systems and other receptors. Many of these molecules are heavily glycosylated, as are the integral proteins, the glycophorins; sialic acid, which comprises the main side-chain of the glycophorins, contributes the most to the negative surface charge of the erythrocyte. Some of these surface proteins are linked to the membrane by the glycosylphosphatidylinositol (GPI) anchor, which provides the hydrophobic domain required for association with the inner hydrophobic part of the membrane. Somatic mutations in the gene for phosphatidylinositol glycan A (*PIGA*) leads to a failure to produce the anchor and to paroxysmal nocturnal haemoglobinuria (discussed in Chapter 31).

The integral proteins and vertical interaction

The two major integral proteins that span the lipid bilayer are band 3 (the anion channel protein) and glycophorin C. Band 3 and associated molecules, 4.2 (pallidin) and ankyrin (2.1), form one major vertical interactive pathway with binding to the β-chain of the spectrin tetramer through ankyrin. Glycophorin C and protein 4.1 also provide a vertical interaction, but the association with spectrin is through a link with actin, which is a key part of the horizontal network. The band 3–4.2–ankyrin– spectrin complex is a central part of the organisation of the lipid bilayer and loss of part of this complex leads to loss of membrane, thereby reducing the surface-area-to-volume ratio of the red cell and leading to the characteristic spherocytes of hereditary spherocytosis.

The main protein of the membrane skeleton is spectrin, consisting of two subunits, α and β, which associate side by side to produce a heterodimer. The dimers associate head-to-head to form tetramers which when fully extended are ~200 nm long. The tail end of the dimer makes contact with a short actin filament composed of 14 monomers; the interaction between spectrin and actin is stabilised by protein 4.1, adducin and dematin. Binding of spectrin dimers to actin filaments produces the more or less hexagonal network of spectrin tetramers on the inner surface of the membrane associated with the lipid bilayer. Spectrin–actin–4.1 interactions provide much of the flexibility of the red cell membrane. Spectrin qualitative defects that affect these horizontal interactions

Table 16.2 Proteins of the red cell membrane.

Band*	Protein	Gene location	Function	Associated haemolytic anaemias
1	α-Spectrin	*SPTA1*, 1q21	Membrane skeletal network	HE, HS
2	β-Spectrin	*SPTB*, 14q24.1–q24.2	Membrane skeletal network	HPP HE, HS
2.1	Ankyrin	*ANK1*, 8p21.1–11.2	Vertical contact	HS
2.9	Adducin	*ADD1* (α-chain), 4p16.3; *ADD2* (β-chain), 2p13.3	Promotes spectrin binding to actin, binds Ca^{2+}/calmodulin	(HS, HE in mice)
3	Band 3. Solute carrier family 4 (anion exchanger) member 1	*SLC4A1*, 17q12–q21	Anion exchange channel, binds glycolytic enzymes	HS, SAO, HAC
4.1	Protein 4.1	*EPB41*, 1p33–p32	Stabilises spectrin–actin contact	HE
4.2	Protein 4.2 (pallidin)	*EPB42* (*PLDN*), 15q15–q21	Band 3–ankyrin complex	HS (Japan)
5	β-Actin	*ACTB*, 7p15–p12	Spectrin network junction	?
6	Ga3PD	*GAPDH*, 12p13.31	Links ATP production to membrane	?
PAS-1†	Glycophorin A	*GYPA*, 4q28–q31	MN blood groups	?
PAS-2	Glycophorin C	*GYPC*, 2q14–q21	Gerbich blood groups	HE
PAS-3	Glycophorin B	*GYPB*, 4q28–q31	Ss blood groups	?

HAC, hereditary acanthocytosis; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; HS, hereditary spherocytosis; SAO, Southeast Asian ovalocytosis; Ga3PD, glyceraldehyde-3-phosphate dehydrogenase.
* Band numbers refer to the position on SDS-PAGE electrophoresis.
† Periodic acid–Schiff stain: bands seen only on PAS-stained gels.

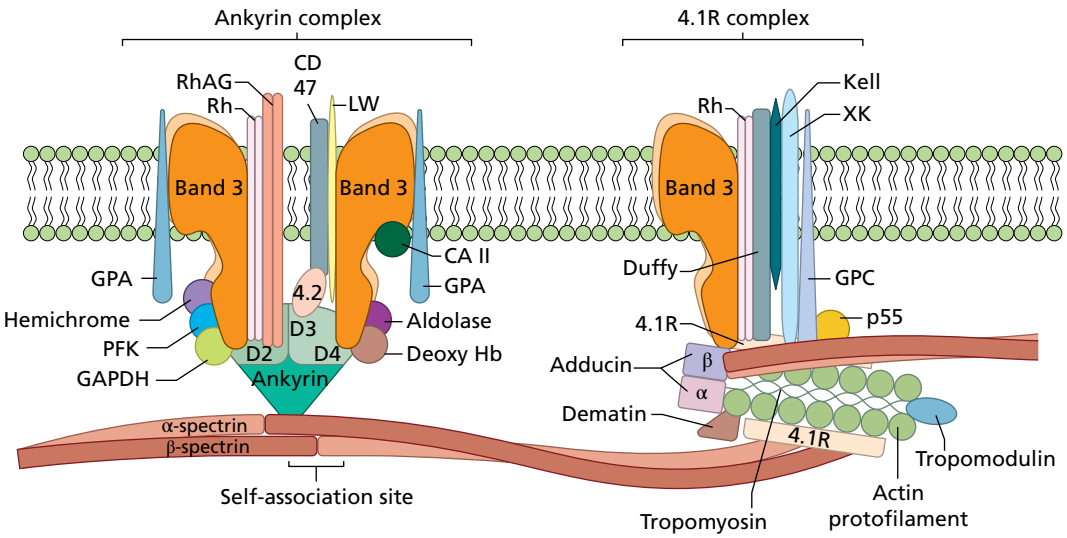


Figure 16.3 A schematic model of the structural organisation of the red cell membrane. The membrane is a composite structure in which the lipid bilayer is linked to the spectrin-based membrane skeleton. The linking of the lipid bilayer to the membrane skeleton is mediated by band 3-ankyrin-protein 4.2– β-spectrin interactions and by glycophorin C–protein 4.1R interaction (vertical interactions).
The horizontal interactions in the spectrin network involve spectrin dimer–dimer interaction and spectrin–actin–protein 4.1 interaction.

tend to induce a loss of structural stability of the membrane and elliptocytosis.

The clinical phenotypes of hereditary membrane disorders

Mutations in the genes that control the proteins of the membrane and their interaction mainly produce changes in the shape of red cells, which is characteristic of any individual. Many of the conditions are inherited as autosomal dominant disorders, homozygosity for major defects mainly being lethal. Severe, bizarre or unexpected red cell morphology is often produced by double heterozygosity or inheritance of more than one defect of the membrane proteins. Mutations affecting the red cell membrane are many and heterogeneous, but the effect on the phenotype can be classified in five main categories: (i) hereditary spherocytosis; (ii) hereditary elliptocytosis and hereditary pyropoikilocytosis (severe form of elliptocytosis); (iii) Southeast Asian ovalocytosis; (iv) hereditary acanthocytosis and (v) hereditary stomatocytosis.

Hereditary spherocytosis

As the name implies, hereditary spherocytosis (HS) is a genetically determined haemolytic anaemia characterised by the spherical shape of the affected red cells. The spherical shape produces a characteristic appearance in the stained blood film of round cells with smaller than normal diameter, which lack the area of central pallor of the normal biconcave discs (Figure 16.4). The disorder is generally inherited as a dominant condition with a wide spectrum of severity. The usual clinical picture is of mild to moderate haemolytic anaemia, but varies from severe neonatal haemolysis with kernicterus (rare) to clinically silent and asymptomatic (usual) haemolysis. Autosomal recessive inheritance occurs in a few mutations, often producing severe haemolysis. In detailed analysis of white populations, HS is one of the most common haemolytic anaemias due to membrane defects, with a prevalence of clinically apparent disease of 200–300 per million population. The occurrence of clinically silent cases probably means that the overall prevalence is likely higher. However, it has been reported in all regions of the world.

Clinical features

The commonest forms of HS present as mild anaemia and jaundice, with a modestly enlarged spleen. However, the genetic heterogeneity of HS (see below) is reflected in the clinical presentation. As the main site of increased red cell destruction in HS is the spleen, it is not surprising that the size of the spleen tends to reflect the severity of the haemolysis, although splenomegaly is rarely marked, enlargement below the umbilicus being very uncommon. When HS presents in adolescence or adult life, it needs to be distinguished from other causes of spherocytosis, particularly warm autoimmune haemolytic anaemia.

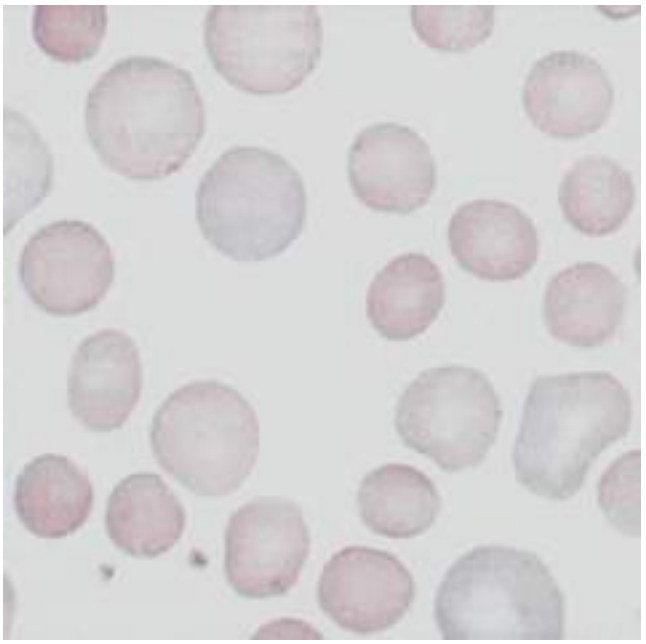


Figure 16.4 Hereditary spherocytosis, peripheral blood. Small spherocytic red cells lack area of central pallor. Large polychromatic red cells (reticulocytes) result in normal MCV, although MCHC may be increased.

HS may present at birth. The functions of the spleen become mature only after birth, so severe anaemia *in utero* is rare. Erythropoiesis is highly active before birth, but enters a phase of reduced activity in the neonatal period. Severe anaemia, developing over 5–30 days post delivery and requiring transfusion, may result from this double physiological development of reduced production and increased destruction, but the anaemia may greatly reduce during the first year of life as compensatory erythropoiesis develops. Decisions about splenectomy do not need to be taken during this time.

Molecular pathology

About 60% of HS cases result from a defect in the ankyrin–spectrin complex, with the genes for ankyrin (*ANK1*) and the α- and β-subunits of the spectrin dimer (*SPTA1*, *SPTB*) being implicated in different genetic types (Table 16.2). A further 25% involve deficiency in band 3, the anion channel. In the remainder of the dominantly inherited HS families, there is a deficiency of protein 4.2 or no abnormality has yet been identified. Deficiency of protein 4.2 is particularly common in Japanese families with HS (Table 16.2). The rare cases of Rh null also present with spherocytosis and mild anaemia. These defects involving spectrin– ankyrin–band 3 interactions affect the vertical interactions.

Laboratory diagnosis

The typical findings of extravascular haemolysis are present in HS (Table 16.1). The diagnosis is usually made on the basis of red cell morphology, backed up where possible with a family history. The mean corpuscular haemoglobin concentration (MCHC) is often increased above 350 g/L, but the presence of macrocytic reticulocytes usually results in a low normal mean corpuscular volume (MCV) rather than true microcytosis. These changes result not only from the reduction of the surface-area-to-volume ratio, but also from the slight dehydration of HS cells. A number of variants of the typical HS features have been described, usually the more severe forms that may have denser and less perfectly round cells in the peripheral blood. In infancy, the morphology may be more difficult to interpret. The effect of immature splenic function and the macrocytosis and anisocytosis of infancy combine with the HS phenotype to produce red cell appearances not typical of the developing HS. Family studies can assist in the diagnosis.

Osmotic fragility test

The osmotic fragility test, which measures the sensitivity of red cells to lysis *in vitro* to swelling caused by incubation in increasingly hypotonic saline solutions, is the most appropriate test to diagnose HS, although its sensitivity decreases in milder cases. In normal red cells with the biconcave disc shape, 50% lysis occurs when the saline solution reaches about 0.5% sodium chloride. The HS cells have less ability to swell and so lyse at higher salt concentrations, producing a right-shifted osmotic fragility curve. One of two patterns may be seen in HS: a generally right-shifted curve, which is the more common finding, and one where there appears to be a 'tail' of lysis-sensitive cells. Incubation of blood for 24 hours at 37 °C accentuates the fragility (Figure 16.5). An alternative to the osmotic fragility test is to measure the extent of membrane surface area loss by flow cytometry following labelling with eosin-5-maleimide (EMA binding test). EMA binds to band 3, which is selectively lost during loss of membrane surface area in red cells from HS patients. The EMA binding screening test has to be used in conjunction with morphology because Southeast Asian ovalocytosis, congenital dyserythropoietic anaemia type II, and cryohydrocytosis also give reduced fluorescence because they can create a long-range modulation effect on the dye binding site in band 3 protein.

Identification of protein abnormalities or gene defects

Methods that identify the defective gene or its product are the most specific for membrane defects but are beyond the scope of most routine haematology laboratories. The original identification of membrane proteins using sodium dodecyl sulfate-solubilised polyacrylamide gel (SDS-PAGE) electrophoresis has led to the classification according to the banding system indicated in Table 16.2 The identification of specific genetic abnormalities may be important in compound haemolytic syndromes, but requires specialised laboratories.

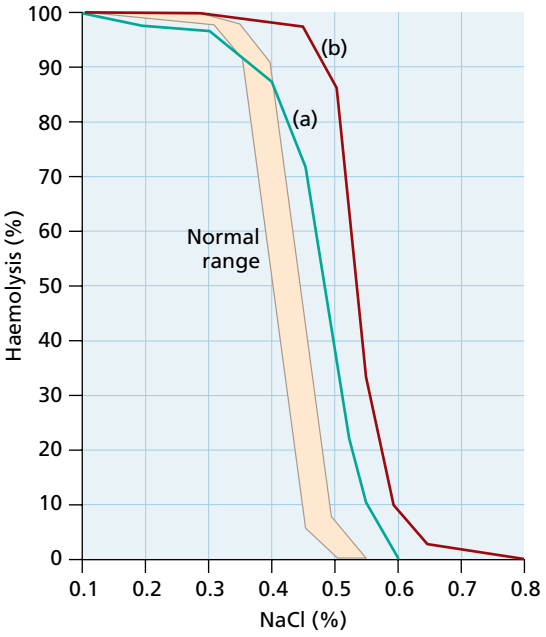


Figure 16.5 Osmotic fragility test in hereditary spherocytosis. Osmotic fragility is increased in the microspherocytes (right shift), but there is also a small population of resistant cells due to increased reticulocytes (a). After splenectomy, the microspherocytes remain, but the proportion of reticulocytes is reduced to normal values and the resistant cells are not seen (b).

Clinical course and complications

In most kindreds, the course of the disorder is similar in affected members although, as with most inherited defects, there is some variable penetrance and it is not rare to find a very mildly affected parent with more severely affected offspring. As with all congenital haemolytic anaemias, the anaemia may be aggravated by environmental factors. This may be consequent of an increase in the red cell destruction or a decrease in production. Increased jaundice may occur during viral infections or bacterial sepsis, the anaemia also being aggravated by a decrease in production consequent on the effects of the acute-phase response or the inhibition of erythropoiesis by interferon (IFN)-γ.

Primary infection with parvovirus (erythrovirus) B19 produces a specific and marked inhibition of erythropoiesis, often characterised as an aplastic crisis. In patients with shortened red cell survival, severe anaemia may be produced by the inhibition, which lasts for some 4–7 days. In normal individuals with a red cell lifespan of 120 days, such an inhibition produces no clinical effect. The anaemia associated with parvovirus infection in HS may require urgent transfusion.

The diagnosis is made by finding absent parvovirus antibodies with subsequent appearance of IgM antibodies. The presence of IgG antibodies at the time of the anaemia excludes the diagnosis.

Acute anaemia due to splenic sequestration is a relatively uncommon complication of HS in childhood. The pathogenesis is probably increased splenic size and activity leading to increased trapping of HS cells within the spleen. This complication may also require urgent transfusion. Malnutrition may increase anaemia because of folate deficiency, but also from increased jaundice through the effect of low-calorie input on unconjugated bilirubin levels in the blood. The anaemia of pregnancy may aggravate a haemolytic anaemia and hence bring the condition to the attention of clinicians and patients. Classical HS is not a risk to mother or child in pregnancy.

Gallstones are an expected complication in HS, as in other chronic haemolytic anaemias. Silent gallstones require no intervention. Recurrent cholecystitis or biliary colic may require cholecystectomy accompanied by splenectomy (see below). Leg ulcers are a rare but well-recognised complication of HS, as with other chronic haemolytic anaemias. Extramedullary haemopoietic masses, usually paravertebral, occur rarely in more severe HS.

Management

Patients with well-compensated haemolysis and no transfusion requirements need no treatment other than reassurance and folic acid supplements (e.g. 400 µg daily or 5 mg weekly). For people with a well-balanced and adequate diet, folic acid supplements are probably unnecessary, but custom dictates the practice should be continued. Radiolucent gallstones, if detected by chance on ultrasound, are common and need no treatment unless complications arise. Gallstones without recurrent inflammation are not a risk factor for carcinoma of the gallbladder. Recurrent cholecystitis or obstruction would be an indication for cholecystectomy, which would also be an indication for splenectomy.

Splenectomy

For the great majority of patients with autosomal dominant forms of HS, splenectomy increases the lifespan of the HS red cells to near normal and alleviates the haemolysis and hyperbilirubinaemia, since the spleen is responsible for the removal of spherocytic red cells with reduced surface area. However, splenectomy carries short- and long-term risks that must be weighed against the benefits in any individual patient. After splenectomy, the blood film continues to show spherocytosis together with common changes associated with splenectomy. The osmotic fragility remains increased.

Risks of splenectomy

The immediate risks associated with splenectomy include those of any abdominal operation together with an increased risk of thrombosis, associated with a marked rise in platelet count that occurs promptly after splenectomy. In HS, in which the erythropoietic drive returns to normal following splenectomy, the platelet count also returns to normal and the risk diminishes. In conditions where haemolysis persists, the platelet count remains elevated, sometimes markedly, and the increased risk of thrombosis continues.

The major hazard of splenectomy is the long-term susceptibility to severe infection, so-called overwhelming postsplenectomy infection (OPSI). The spleen plays an important role in filtering and phagocytosing bacteria and removing parasitised erythrocytes from the blood. The spleen is the major source for mounting the rapid, specific IgM response to organisms that enter through the gut. The main organisms of this class are the encapsulated organisms, *Streptococcus pneumoniae*, *Haemophilus influenzae* type B and *Neisseria meningitidis*. Pneumococcal infection is responsible for about 70% of OPSI and has a 60% mortality. Lack of a spleen greatly increases the virulence of the infection, with progression from the first feeling of fever and non-specific flu-like symptoms to irreversible endotoxic shock occurring in a matter of hours. Patients may present with purpura, evidence of disseminated intravascular coagulation, multiorgan failure, hypotension and peripheral limb ischaemia. Diarrhoea and vomiting are common prodromes. It is this speed of progression that makes the prophylaxis of this fortunately uncommon complication so important. Prophylaxis depends on education and awareness for the patient, specific measures to reduce the risk from particular organisms and the provision of information concerning the splenectomy for healthcare workers (Table 16.3). There is no direct evidence that phenoxymethylpenicillin (e.g. 250 mg twice daily) reduces the risk of OPSI in splenectomised patients, but good evidence that it does so in homozygous sickle cell patients who have functionally inactive spleens. It is on this evidence that such antibiotic prophylaxis (or erythromycin 250 mg b.d. for those sensitive to penicillin) is recommended. The actual incidence of OPSI is difficult to calculate. The overall risk has been stated as 0.04 per 100 patient-years for patients without added immunosuppression, but considerably higher for those immunocompromised by malignancy or chemotherapy. The risk is greatest in the first 2 years after splenectomy but continues lifelong. Children under the age of 5 years are particularly susceptible and splenectomy should be avoided in this group if at all possible.

Indications for splenectomy

Patients with marked haemolysis producing symptoms or requiring transfusion should be splenectomised, although preferably not before the age of 5 years (later if possible). Recurrent aplastic crises are also an indication. Attacks of cholecystitis or biliary colic warrant cholecystectomy and splenectomy, but symptomless gallstones are not a necessary indication. In small children with severe anaemia, partial splenectomy has been used to decrease the extent of anaemia, but

Table 16.3 Guidelines for prevention and management of infection in the splenectomised patient.

It is essential to educate patients regarding the risk and the importance of prompt recognition and treatment of infections. Leaflet card for patients to alert health professionals to risk of OPSI.
All patients should receive polyvalent pneumococcal, *Haemophilus influenzae* type b and meningococcal vaccination.
Vaccines should ideally be given 2 weeks prior to splenectomy or 2 weeks after splenectomy.
Pneumococcal immunisation
- Infants <2 years*: should be immunised with three doses at the national schedule with polyvalent-PCV vaccination. One dose of PPV at 2 years should also be offered.
- Children 2–5 years: one dose of PPV or PCV13 followed by PPV depending on previous routine childhood vaccinations.
- Children >5 years and adults: irrespective of immunisation status should continue to receive one dose of PPV by measuring antibody response.
- Responders should be revaccinated with PPV at 5-year intervals.

Haemophilus influenzae b (Hib) vaccination
- Infants <2 years: should complete their vaccination according to the national schedule.
- Children >2 years and adults: should be offered one dose of Hib-containing vaccine irrespective of their previous immunisation status.
- Meningococcal vaccination (Men)
- The quadrivalent MenACWY conjugate vaccine is recommended in preference to the plain polysaccharide meningococcal vaccine for all age groups.
- Infants <2 years, if immunised or partially immunised: 3 and 4 months of age; MenC conjugate vaccine at 12 months of age as well as quadrivalent MenACWY no earlier than 1 month later. At 2 years an additional booster of Hib/MenC should be given at second birthday.
- Children >2 years and adults: should receive one dose of MenC conjugate vaccine plus a single dose of quadrivalent MenACWY conjugate vaccine 1 month later.
- Travellers to endemic areas should receive quadrivalent MenACWY conjugate vaccine before travelling.

All patients should receive yearly influenza vaccination.
Lifelong prophylactic antibiotics (oral phenoxymethylpenicillin or an alternative).
Patients developing infection despite measures should receive systemic antibiotics and be admitted urgently to hospital.
Awareness of risks of malaria and scrupulous prophylaxis if at risk.

PCV, vaccination pneumococcal conjugate vaccines; PPV, pneumococcal polysaccharide vaccine; PCV13, 13-valentPCV.
* Age at which splenectomy is performed.
(*Source:* Davies *et al.*, *Brit J Haemat* **155**: 308–17, on behalf of the Working Party of the Haemato-Oncology Task Force of the British Committee for Standards in Haematology (2011).)

the performance of this procedure requires special surgical skills and there is need for careful long-term follow-up.

Hereditary elliptocytosis

Deficiency of spectrin tetramers, the horizontal links of the cytoskeleton, produces a wide spectrum of disease from fully compensated haemolysis with mildly elliptocytic red cells to severe and life-threatening anaemia with grossly distorted cells. When the morphological characteristic is a relatively uniform elliptical shape, the condition is referred to as hereditary elliptocytosis (HE). Haemolytic anaemia associated with the more distorted forms, which are also heat labile, was previously called hereditary pyropoikilocytosis (HPP). Recent evidence implies that HPP is a severe form of HE. Within a family, HE and HPP may both be present, the more severely affected individuals having both spectrin deficiency as well as a relative deficiency of spectrin tetramers. This may be caused by coinheritance of a low-expression allele for α-spectrin, compound heterozygosity for two HE alleles or HE homozygosity. A number of families have been described in which mutations involving the protein 4.1 gene result in failure to produce the protein. In heterozygotes with this variant, elliptocytosis occurs without haemolysis; in homozygotes, there is a severe haemolysis with extensive cell fragmentation.

Clinical features

As with HS, haemolytic anaemia in HE has a heterogeneous clinical presentation and molecular basis. The anaemia can range from very mild to severe with splenomegaly and few cases of hydrops fetalis have been reported. The clinical management of HE parallels that of HS.

Mild common hereditary elliptocytosis

Frequently, HE is discovered by chance from a blood film (Figure 16.6) or the presence of marginally raised bilirubin. Some affected people have no evidence of shortened red cell survival, whereas others have a well-compensated haemolytic anaemia.

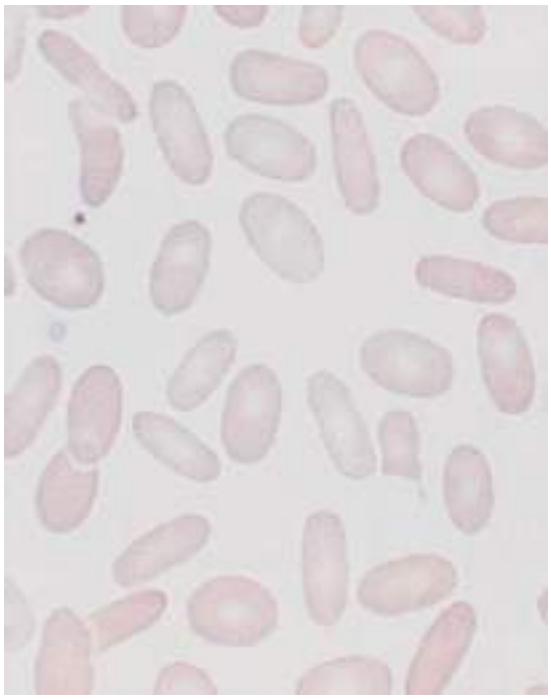


Figure 16.6 Hereditary elliptocytosis, peripheral blood. Characteristic elliptocytes of mild common hereditary elliptocytosis (Source: Haemolytic Anaemia.)

No treatment is required, although the blood film of partners should be examined if there is consanguinity, making homozygosity in offspring possible. For patients with mild haemolysis, anaemia may increase during infections, in pregnancy, with folate deficiency or with other conditions likely to enhance anaemia.

Silent carriers: low-expression genes

Mutations that produce low expression of α-spectrin may lead to no haematological abnormality because of the normal overexpression of α-spectrin in red cells compared with β-spectrin. However, when these defects are inherited in *trans*, on the other allele from an HE gene, HPP may result. Several mutations have been described, particularly commonly in codon 28, which produces low-expression genes. A common polymorphism, intron 45C→T, is spectrin α^{LELY} (LELY denoting 'low-expression allele Lyon').

Haemolytic hereditary elliptocytosis

The characteristics of haemolytic HE previously termed HPP are densely contracted and fragmented cells (Figure 16.7), moderate to severe haemolysis and heterogeneity of manifestations within a family. In general, patients with haemolytic HE have spectrin deficiency in addition to the abnormalities of spectrin–spectrin contacts resulting in tetramer deficiency. One parent of an HE propositus may have normal haematology

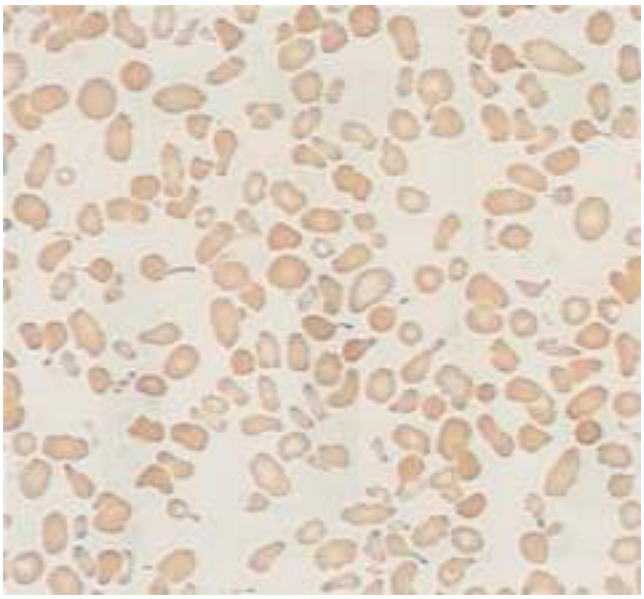


Figure 16.7 Hereditary pyropoikilocytosis, peripheral blood. Marked anisocytosis and poikilocytosis from a child with homozygous hereditary elliptocytosis.

but carry a mutation in *trans*, which leads to spectrin deficiency. The affected cells show thermal lability and fragmentation at lower temperatures than normal, and erythrocytes are mechanically unstable.

Hereditary elliptocytosis and poikilocytosis in the neonate

In the neonate, the manifestations of HE may be a more marked poikilocytosis resembling HPP, with more fragmented red cells. These red cells are susceptible to fragmentation above 46 °C, whereas normal cells only fragment above 50 °C. The morphological changes and haemolysis gradually decrease over the first year until the typical picture of mild HE remains. Treatment of neonatal poikilocytosis is required only if the anaemia is such as to warrant transfusion.

Laboratory investigation

The standard approach to the diagnosis of HE is the identification of haemolysis, coupled to a careful examination of the blood film of the patient and as many first-degree relatives as possible. Examples of blood films are shown in Figures 16.6 and 16.7. Other acquired causes of elliptocytic or fragmented red cells need to be excluded, including iron, folate or vitamin B_{12} deficiency and the microangiopathic haemolytic anaemias. Congenital dyserythropoietic anaemia and thalassaemia intermedia also need to be excluded.

As with the investigation of HS, appropriate gel electrophoresis assays may reveal protein abnormalities, although more specific

identification requires a sophisticated approach beyond the abilities of most haematology laboratories.

Treatment

Patients with chronic haemolysis should be given folate supplements. Splenectomy is indicated for severe haemolytic anaemia in patients with haemolytic HE. Response in HE may not be complete, but the anaemia is usually markedly alleviated. As with HS, there may be an increased risk of increased thrombotic tendency. The precautions against OPSI are the same as for HS.

Hereditary stomatocytosis and related disorders

Stomatocytes are so-called from the mouth-like slit or 'stoma' that appears on blood films (Figure 16.8). Stomatocytes are leaky to cations. There are other variations with Na^+ or K^+ leaks that are clinically similar to stomatocytosis without the obvious morphological changes. All these conditions are inherited in autosomal dominant fashion and mostly produce moderate haemolytic anaemia (haemoglobin 100 g/L or above) and macrocytosis. There are two main variants: over-hydrated hereditary stomatocytosis associated with mutations in *RHAG* gene, in which MCHC is low, and dehydrated hereditary stomatocytosis, with an increased MCHC; this latter including hereditary xerocytosis due to abnormalities of mechanosensitive channel *PIEZO1* and Gardos channelopathy (*KCNN4* variants). Other rare variants of stomatocytosis are pseudohyperkalaemia and cryohydrocytosis with neurological impairment, respectively due to defects in *ABCB6* or *SLC2A1* genes.

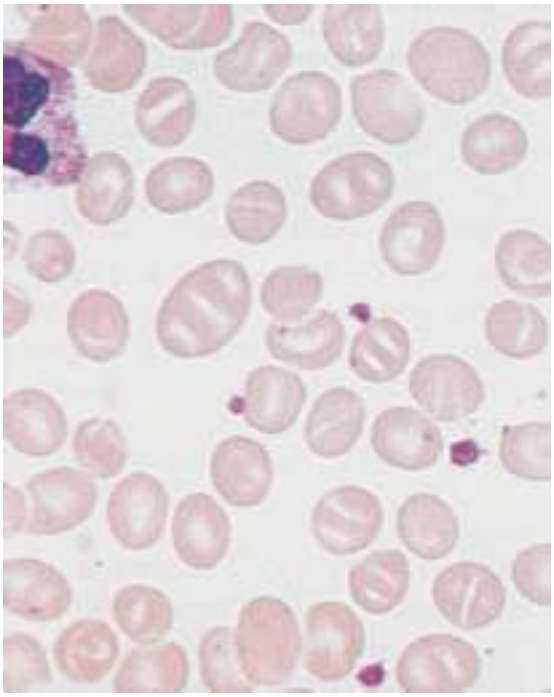


Figure 16.8 Hereditary stomatocytosis, peripheral blood.

Table 16.4 Features of hereditary stomatocytosis and related disorders.

Characteristic	Expression	Group affected
Haemolytic anaemia	Mild to moderate	All variants
	Absent	Hereditary pseudohyperkalaemia
Morphology	Macrocytosis	All variants
	Stomatocytosis	Variable
MCHC	Decreased	Over-hydrated HSt (hydrocytosis)
	Increased	Dehydrated HSt (hereditary xerocytosis, desiccocytosis)
Serum [K^+]	Raised *in vitro*	Pseudohyperkalaemia Dehydrated HSt Cryohydrocytosis
Thrombotic tendency	Post splenectomy	All variants
Fluid balance	Perinatal oedema	Dehydrated HSt

The blood film may show stomatocytosis, but more commonly the film is unremarkable, apart from macrocytosis and polychromasia. The group is rare, estimates suggesting that 1 in 10,000–1 in 100,000 of the population are affected. However, associated features make the conditions important beyond their rarity (Table 16.4). Pseudohyperkalaemia may occur because K^+ leaks rapidly from the red cells at room temperature. In some individuals, there is no evidence of haemolysis, only macrocytosis and pseudohyperkalaemia. Unless the cause of the apparent hyperkalaemia is diagnosed, unnecessary, and even dangerous, investigation and treatment may be undertaken. In some families, the K^+ leak is greatly increased *in vitro* by cold (cryohydrocytosis). In dehydrated hereditary stomatocytosis, there may be marked perinatal ascites that resolves spontaneously over the first year of life, but which again can lead to extensive unnecessary investigation. The third problem with hereditary stomatocytosis, both over-hydrated and dehydrated varieties, is that splenectomy is followed by very marked thrombotic tendencies such that splenectomy should not be performed.

Laboratory investigations

Tests for haemolysis and examination of the blood film of the patient and close relatives are the first steps in diagnosis.

The finding of a raised serum potassium, together with macrocytosis, especially with some evidence of haemolysis, indicates the pseudohyperkalaemia of dehydrated hereditary stomatocytosis. Additional studies involve the measurement of intracellular [Na^+] or [K^+] and their flux through the membrane at different temperatures. Four subgroups have been defined according to the intracellular sodium concentration (normal 5–10 mmol/L). Patients with the pseudohyperkalaemia of dehydrated hereditary stomatocytosis may have normal or slightly high sodium concentrations (12–18 mmol/L); in families with cryohydrocytosis, (temperature-sensitive leak) the sodium concentration is 20–50 mmol/L, and in over-hydrated hereditary stomatocytosis, the sodium concentration is 60 mmol/L or more.

Osmotic gradient ektacytometry analysis that provides simultaneous information on RBC geometry, cytoplasmic viscosity, volume regulation and fluidity of the membrane has been considered for many years the gold standard for the diagnosis of this group of disorders. By this technique, dehydrated stomatocytosis due to *PIEZO1* gene mutations is characterised by a left-shifted profile, and over-hydrated stomatocytosis by a right-shifted profile. Of note, in Gardos Channelopathy, most patients display a normal ektacytometric curve.

Recently the molecular basis of hereditary stomatocytosis has been elucidated, and the genes involved (Table 16.5) are usually included in targeted-next generation sequencing diagnostic panels of hereditary haemolytic anaemias. Consequently the identification of the molecular lesion is now considered mandatory to confirm the diagnosis.

Treatment

There is rarely a need for measures to raise the haemoglobin, and splenectomy should be avoided because of the risk of thrombosis, including hepatic and portal vein thrombosis. If splenectomy is necessary, lifelong anticoagulation should be considered.

Southeast Asian ovalocytosis

A dominantly inherited ovalocytosis is found in parts of Southeast Asia, where Plasmodium *falciparum* malaria is common, particularly in Papua New Guinea, Borneo, Southern Thailand and the Philippines. The red cell morphology is ovalocytic rather than elliptocytic. Stoma-like slits may be present and transverse banding in the red cells is seen (Figure 16.9). Most individuals have no haemolysis except in the neonatal period but, in a few, mild anaemia may be present. The molecular defect is a deletion of nine amino acids at the transmembrane cytosol junction of band 3; the defect possibly limits the mobility of band 3 within the membrane. Homozygosity is rarely found and is presumably lethal *in utero*.

Abnormalities of membrane lipids

Acanthocytosis

Acanthocytes, or spur cells, show prominent, somewhat regular projections on the surface, best demonstrated by scanning electron microscopy. They are formed when the outer lipid layer of the membrane acquires additional lipid. Acanthocytosis is an acquired characteristic of severe liver disease, usually end stage, and the result of interaction of altered plasma lipids.

Table 16.5 Molecular basis of hereditary stomatocytosis and related disorders.

Disease	Defective protein	Gene	Chromosome	Clinical phenotype
Hereditary xerocytosis	FAM38A Mechanosensitive ion channel	*PIEZO1*	16q24.3	Haemolytic anaemia mild-moderate Polycythaemia
Gardos channelopathy	KCa3.1 – Gardos channel	*KCNN4*	19q13.31	Haemolytic anaemia mild-moderate
Overhydrated stomatocytosis	Rh associated glycoprotein	*RHAG*	6p21.1-p11	Haemolytic anaemia
Cryohydrocytosis	Glucose transporter 1 – GLUT1	*SLC2A1*	1p35-p31.3	Haemolytic anaemia Neurologic symptoms (rare cases)
Familial pseudohyperkalaemia	ATP binding cassette subfamily B	*ABCB6*	2q.36	Pseudohyperkalaemia No anaemia
Sitosterolaemia	ATP binding cassette subfamily G	*ABCG5* *ABCG8*	2p.21	Sitosterols accumulation Haemolytic anaemia Macrothrombocytopenia
South East Asian Ovalocytosis	Anion exchange channel – Band 3	*SLC4A1*	17q21.31	Haemolytic anaemia mild/ compensated

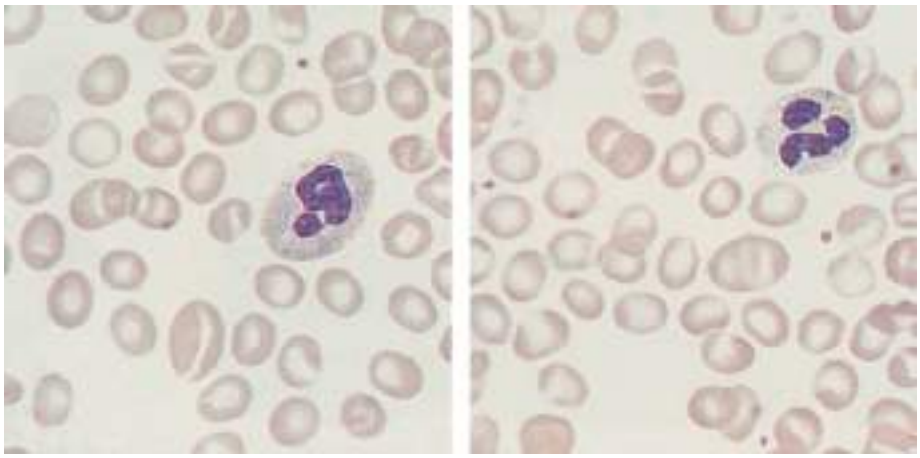


Figure 16.9 Southeast Asian ovalocytosis, peripheral blood films. Mild ovalocytosis and some stomatocytosis. Some cells have apparent transverse ridge.

Abetalipoproteinaemia

Abetalipoproteinaemia is a rare inherited defect with absent β-apolipoprotein, which results in low serum cholesterol, but increased sphingomyelin, which enters the cell membrane and produces the acanthocytes. The main clinical features are retinitis pigmentosa, fat malabsorption and hepatic encephalopathy.

McLeod phenotype

In the McLeod phenotype, acanthocytosis occurs (Figure 16.10), together with decreased expression of the Kell antigen. The defective gene is on the X chromosome (Xp21), close to the genes for Duchenne muscular dystrophy and retinitis pigmentosa, conditions with which the phenotype has been linked. The gene codes for the Kx protein and is linked to the Kell blood group protein in the membrane. There may be mild anaemia.

Defects of red cell metabolism

The main function of the red cell is to carry haemoglobin around the circulation in high concentration and in a functional state so that gas exchange may occur efficiently in the lungs and in the tissue capillaries. In order to fulfil its function, the red cell needs a supply of energy in the form of ATP and a source of reducing power.

Mature red cells contain no DNA or RNA and hence are incapable of protein synthesis, and the only source of energy as ATP is derived from anaerobic glycolysis, the linked reducing system of the hexose monophosphate shunt (pentose phosphate pathway) and the glutathione cycle. ATP is required to maintain the membrane in its deformable state, with asymmetric lipid layers, and to regulate ion and water exchange. Reducing power is required to reduce methaemoglobin to its functional state of deoxyhaemoglobin and to counteract the strong oxidative stresses which a circulating cell carrying molecular oxygen encounters. The main process that reduces methaemoglobin utilises reduced nicotinamide adenine dinucleotide (NADH), produced from nicotinamide adenine dinucleotide (NAD^+) by the glycolytic

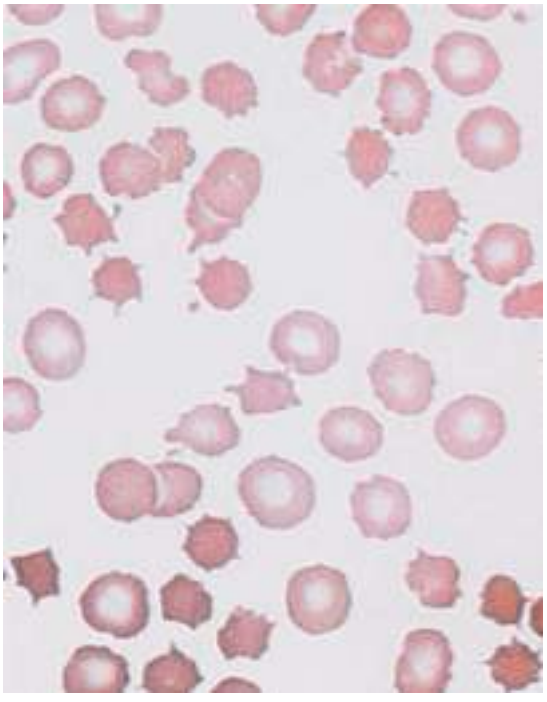


Figure 16.10 McLeod syndrome, peripheral blood. Note the marked acanthocytosis.

pathway. Reduction and detoxification of free oxygen radicals and hydrogen peroxide produced during reactions to infection are provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated by the first steps of the pentose phosphate pathway, catalysed by glucose-6-phosphate dehydrogenase (G6PD) and the linked enzyme 6-phosphogluconate dehydrogenase. NADPH drives the glutathione cycle, glutathione (GSH) being the major reducing agent within the red cell.

The lack of protein synthesis in the mature red cell means that none of the enzymes in the metabolic pathways can be replaced during the red cell's lifespan. Over the 120 days of normal red cell survival, enzyme activities decline at variable but predictable rates. This decline probably contributes to the ageing process of the red cell. Many of the abnormalities that affect red cell metabolism provoke haemolytic anaemia.

The glycolytic pathway (Embden–Meyerhof pathway)

Glycolysis is the process by which glucose is converted to pyruvate through a number of steps, with a net gain of two molecules of ATP generated for each molecule of glucose metabolised. Glucose is derived from the plasma by facilitated transfer through the membrane. Pyruvate and lactic acid are in equilibrium determined by the redox potential of the cell (NAD^+/NADH) and can diffuse out of the cell. The internal milieu of the cell, with its high K^+ concentration and presence of other cations, such as magnesium, necessary for efficient glycolysis, is maintained through the activity of various ion channels in the membrane. The glycolytic pathway also provides the redox reaction to convert methaemoglobin to deoxyhaemoglobin by utilising NADH in a reaction catalysed by methaemoglobin-NADH reductase (cytochrome b_3). The various products and functions of glucose metabolism are shown schematically in Figure 16.11 and in detail in Figure 16.12.

The Rapoport–Luebering shunt

One of the essential roles of erythrocyte metabolism is also to provide sufficient 2,3-diphosphoglycerate (2,3-DPG) to regulate the oxygen affinity of haemoglobin. 2,3-DPG is produced from 1,3-DPG under the influence of the enzyme diphosphoglycerate mutase in linked reactions that form the Rapoport–Luebering shunt: 2,3-DPG is broken down to 3-phosphoglycerate by a phosphatase and thus re-enters the glycolytic pathway. It should be noted that when metabolism takes place via the Rapoport–Leubering shunt, there is bypass of the stage of ATP production (Figure 16.12). The shunt thus not only provides the 2,3-DPG for interaction with the haemoglobin tetramer, but also acts as an energy control mechanism for glycolysis.

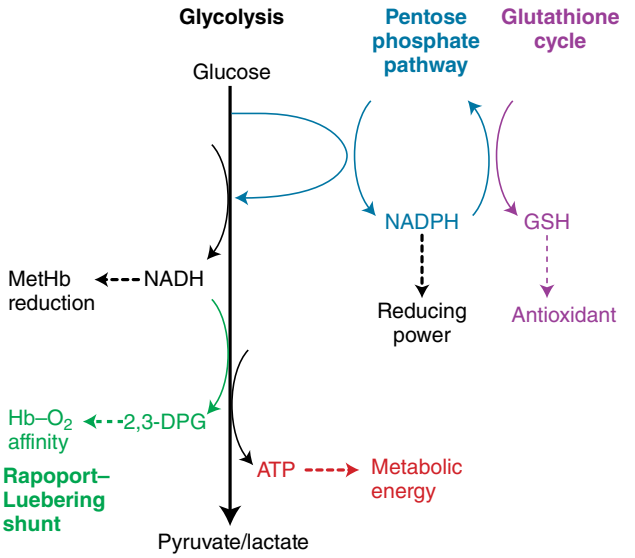


Figure 16.11 Principal pathways of energy production in the mature red cell. The glycolytic pathway provides energy in the form of ATP. Under normal conditions, methaemoglobin (MetHb) is reduced by the coupled reaction with $NADH^+$. The Rapoport–Luebering shunt provides 2,3-diphosphoglycerate (2,3-DPG) for control of haemoglobin oxygen affinity. Reducing power is produced by the pentose phosphate pathway and is linked to redox reactions through the glutathione cycle.

Disorders of the glycolytic pathway

Mutations of most of the enzymes in the glycolytic pathway have been described in association with congenital non-spherocytic haemolytic anaemia (CNSHA), as the result of a failure to produce sufficient ATP. However, when the defective enzyme is expressed throughout the body, other non-haematological symptoms may occur.

Pyruvate kinase deficiency

Pyruvate kinase (PK) is one of the dominant controlling steps in glucose metabolism (together with hexokinase and phosphofructokinase). It catalyses the final steps of the glycolytic pathway with the conversion of phosphoenolpyruvate to pyruvate, with the concomitant phosphorylation of ADP to ATP, leading to overall net gain of ATP from this pathway. There are four tissue-specific isoenzymes codified by two separate genes. The *PKM2* gene, on chromosome 15, produces *PKM1* and *PKM2* through differences in post-transcriptional splicing. *PKM1* is present in skeletal muscles, *PKM2* in leucocytes, kidneys, adipose tissue and lungs. The *PKLR* gene, on chromosome 1, gives rise to PKL in the liver and PKR in red cells, controlled by tissue-specific promoters. The active enzymes are homotetramers. *PKM2*, PKR and PKL demonstrate marked allosteric reactions with several ligands; *PKM1*, on the other hand, has no allosteric interactions.

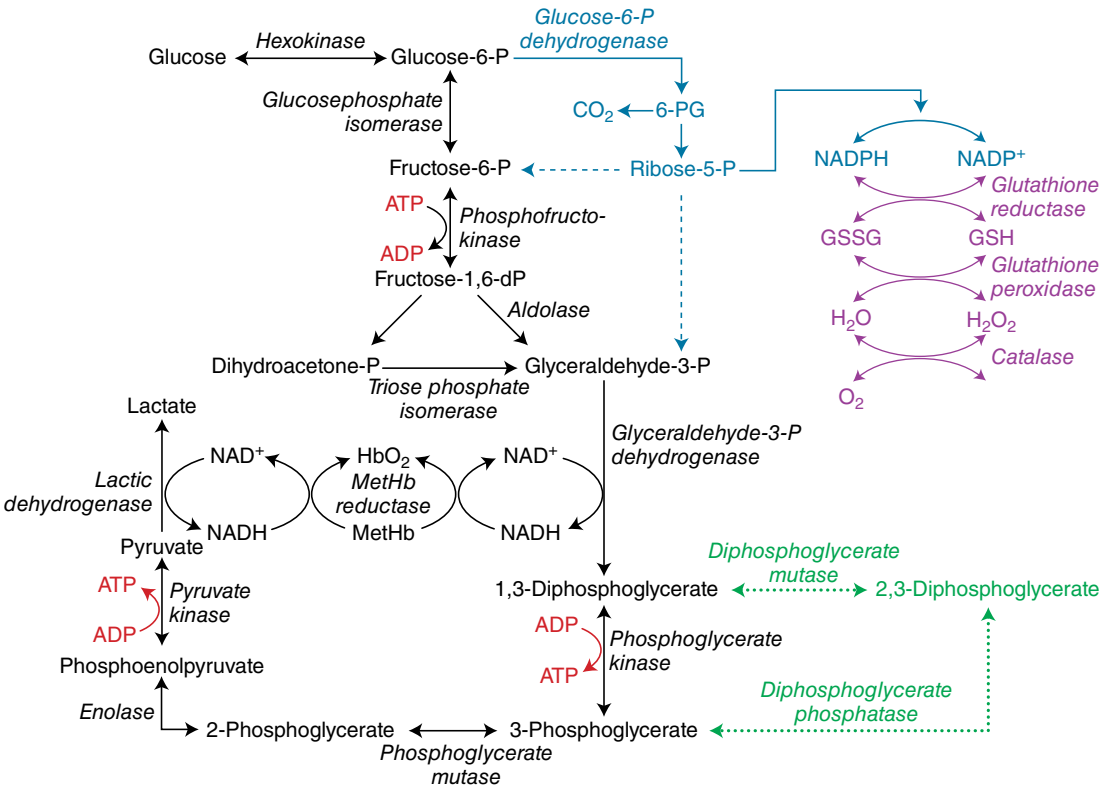


Figure 16.12 The glycolytic pathway and interactions with the other metabolic pathways.

PK deficiency is the most common enzymopathy of the glycolytic pathway inherited in an autosomal recessive form. The prevalence of this disease, as assessed by gene frequency studies, has been estimated to be 1:20,000 in the general white population. More than 250 different mutations have been described in *PKLR* gene, the majority involving missense mutations, a few deletions or mutations in the promoter region. Many individuals are compound heterozygotes. Not surprisingly, there is enormous genetic heterogeneity between affected individuals, reflected in the multiplicity of quantitative and kinetic defects. Because PKR is a homotetramer, the genotype–phenotype correlations are difficult to predict, although by the analysis of an extensive large series of 257 PK deficient patients, it has been shown that patients with homozygous or compound heterozygous non-missense variants (e.g. stop-codon, frameshift and splicing variants) have lower haemoglobin levels, higher numbers of lifetime transfusions and higher rates of complications including iron overload. The comparison of recombinant mutants of human PKR with wild-type enzymes has revealed the effect of amino acid replacements on the molecular properties of the enzyme. However, the clinical manifestations of red cell enzyme defects are not merely dependent on the molecular properties of the mutant protein but rather reflect the complex interactions of additional factors, including genetic background, concomitant functional polymorphisms of other enzymes, post-translational or epigenetic modifications, ineffective erythropoiesis and differences in splenic function.

Deficient PK activity leads to accumulation of substrates further up the pathway, including 2,3-DPG. The increased concentration of 2,3-DPG in PK-deficient red cells shifts the oxygen dissociation curve to the right, indicating low oxygen affinity. It has been reported that PK-deficient patients tolerate apparent anaemia well because the lower haemoglobin content will deliver the same amount of oxygen to the tissues as normal haemoglobin, at least under normal conditions, though oxygen reserves would be limited.

Reticulocytes have alternative means of producing energy in the form of ATP via the oxidative respiratory pathway of the remaining mitochondria. They can also synthesise enzyme in those variants characterised by enzyme instability in mature red cells. Reticulocytes thus have a metabolic advantage over mature red cells in PK deficiency.

Clinical features

The genetic heterogeneity is reflected by the wide variation in the phenotype. The presenting features may vary from severe neonatal jaundice and anaemia, rarely even presenting with hydrops fetalis, severe chronic non-spherocytic haemolytic anaemia requiring repeated transfusions, moderate haemolysis with exacerbation during infections or pregnancy, to symptomless

compensated haemolysis with only a minor apparent anaemia. The majority of reported cases have presented in childhood. PK deficiency does not usually have an adverse effect on the outcome of pregnancy, although occasionally transfusion may be required to compensate for the added dilutional anaemia.

Jaundice, as with other congenital haemolytic anaemias, may be exacerbated by coinheritance of other genes, as in Gilbert syndrome. The haemolysis is nearly always extravascular, though rare examples with some intravascular haemolysis have been detected, particularly in infancy. Gallstones are common in PK deficiency and may lead to bouts of cholecystitis and biliary colic. Jaundice may also be increased by administration of drugs that affect bile excretion. As with other cases of congenital haemolytic anaemia with extravascular haemolysis, excess iron accumulation may occasionally develop, even in the absence of transfusion or coinheritance of a haemochromatosis gene.

The spleen is usually palpable in cases with significant haemolysis, though in milder cases it may only be evident by ultrasound or other imaging techniques.

Laboratory diagnosis

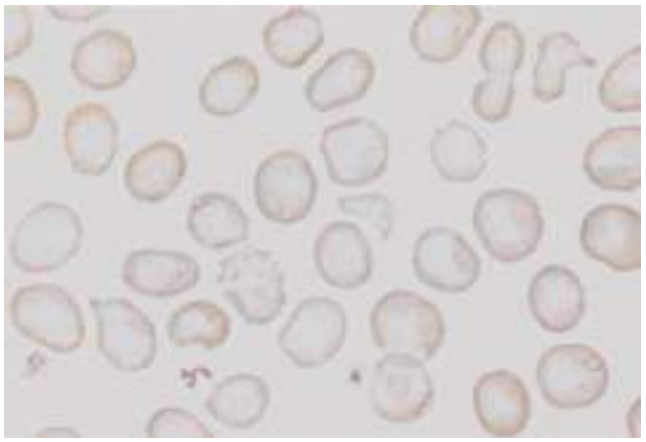
The haematological features of PK deficiency are common to other hereditary non-spherocytic haemolytic diseases; for this reason, the diagnosis ultimately depends on the exclusion of other causes of haemolytic anaemia, upon the demonstration of low enzyme activity and the confirmation at DNA level.

The blood count reveals normochromic anaemia with reticulocytosis, sometimes producing a slight macrocytosis. An increased mean corpuscular haemoglobin concentration is occasionally seen in severe cases due to dehydration brought about by ATP deficiency. Red cell morphology is commonly unremarkable, displaying anisocytosis and a variable portion of spur cells or acanthocytes (which are not specific), particularly after splenectomy. A further increase of reticulocytes postsplenectomy is typically observed (Figure 16.13a–c).

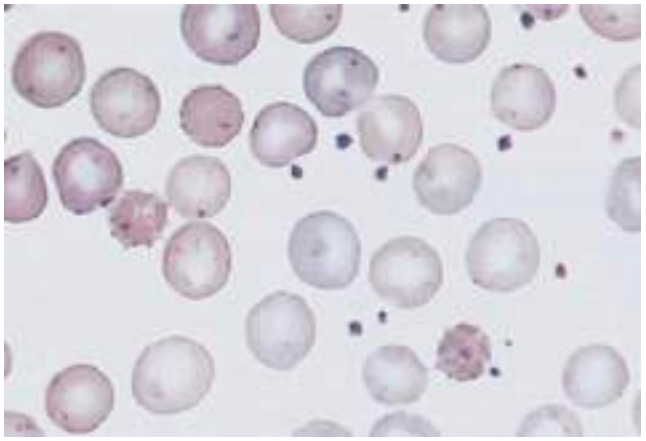
The decreased enzyme activity is associated with an elevated 2,3-DPG level (2–3 times normal). Meaningful enzyme levels can only be achieved after total removal of leucocytes, which have up to 300 times the PK activity of red cells; the effect of reticulocytes also has to be taken into account. No correlation has been described amongst the residual PK activity, degree of haemolysis and clinical severity of PK deficiency.

Management

Red cell transfusions may be required in severely anaemic cases, particularly in the first years of life; the haemoglobin then tends to stabilise in many cases at about 60–80 g/L, and transfusions are no longer necessary unless the anaemia is exacerbated by infections, pregnancy or other conditions. Because the delivery of oxygen to tissues is highly efficient due to the high 2,3-DPG content, the decision to transfuse a PK-deficient patient should be based on the clinical conditions rather than on the haemoglobin levels.



(a)



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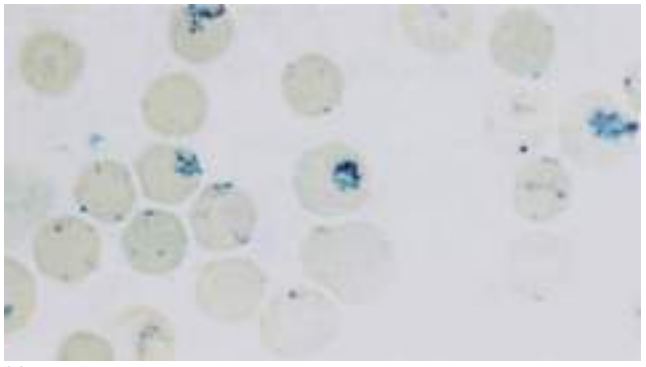

(c)

Figure 16.13 PK deficiency, peripheral blood. (a) Red cell anisocytosis and poikilocytosis before splenectomy. (b) After splenectomy showing acanthocytes or 'prickle' cells. (c) Gross reticulocytosis after splenectomy (supravital new methylene blue stain) (Source: europeanmedical.info.)

Splenectomy does not arrest haemolysis and should be reserved for severely affected young patients who need regular blood transfusions and to patients who do not tolerate anaemia. It usually results in an increase of 10–30 g/L in haemoglobin,

reducing or even eliminating transfusion requirement. Indications and risks of splenectomy, particularly the risk of overwhelming sepsis, are the same reported for red cell membrane disorders. Aplastic or haemolytic crises may still occur after splenectomy. As with all chronic haemolytic anaemias, folic acid 5 mg per week or 400 μg daily is a sensible supplement. Iron chelation may be required since iron overload is not uncommon in PK deficiency, even in non-transfused patients. Bone marrow transplantation has been performed in a limited number of cases and should be considered an investigational treatment: the analysis of 16 PK-deficient patients known to be transplanted to date, showed a three-year overall survival of 65%. Significantly better survival was observed for patients transplanted before the age of ten.

Recently novel therapeutic approaches have been proposed: a small molecule allosteric activator of PK has been developed with safety and efficacy demonstrated in clinical trials. A gene therapy clinical trial for PK deficiency was launched in 2020.

Other defects of the enzymes of the glycolytic system

Compared with PK deficiency, other defects of the glycolytic pathway are very rare. The main features of these disorders are summarised in Table 16.6.

Hexokinase deficiency

Hexokinase catalyses the phosphorylation of glucose to glucose-6-phosphate (G6P), the first step in the glycolytic pathway. The erythrocytic isoenzyme, derived from alternative splicing, differs from that in nucleated cells, which have oxidative respiration, by lacking a porin-binding domain that links the enzyme to the mitochondrial membrane. The enzyme provides a major rate-limiting step in glycolysis and has extensive allosteric interactions: it is highly pH sensitive and its activity is regulated by its products, G6P, P_i, 2,3-DPG and disulfide compounds. The enzyme activity decays predictably with age of the normal red cell and may be used as a comparator for other enzyme activities where absolute levels may be difficult to interpret because of the age distribution of the red cells.

Hexokinase deficiency has been recorded in about 20 cases characterised by molecular and phenotypic heterogeneity. Most patients have moderately reduced activity and complete hexokinase deficiency is probably lethal. The enzyme deficiency results in moderate, mild anaemia, but some cases present with severe anaemia and death in neonatal period. Typically, reduced hexokinase activity is associated with a low concentration of 2,3-DPG within the cells. Patients have less exercise tolerance for a given level of haemoglobin than would be expected because of the left shift in the oxygen dissociation curve.

Glucose phosphate isomerase deficiency

Glucose phosphate isomerase (GPI) catalyses the second step of glycolysis, the interconversion of G6P to fructose-6-phosphate (F6P). The enzyme is also known as phosphohexose isomerase, phosphoglucose isomerase, autocrine motility factor and neuroleukin, indicating that the protein has other actions in other cells.

GPI deficiency is one of the commonest causes of CNSHA after G6PD and PK deficiency. About 30 different mutations have been identified, underlying the molecular heterogeneity and consequently the heterogeneity of the clinical picture. Most reported cases present with mild to moderate haemolytic anaemia; hydrops fetalis may occur. In T lymphocytes, GPI acts as neuroleukin, a lymphokine that induces the formation of antibody-secreting cells. GPI is also expressed in neutrophils, but there is no increase in infections in deficient subjects. In some deficient patients, neurological impairment or intellectual disability has been reported, thought to be related to hypoxia or ischaemia *in utero* rather than to direct metabolic effects.

Phosphofructokinase deficiency

Phosphofructokinase (PFK) catalyses a reaction in which F6P is phosphorylated to fructose-1,6-diphosphate, ATP being the donor of the phosphate group. Under normal physiological conditions, this may be the major rate-limiting step in glycolysis in the red cell. Erythrocytic PFK is a tetramer composed of M or L subunits encoded by two separate genes; there may be five isoenzymes composed of different numbers of L and M subunits. PFK is a homotetramer of M subunits (M4) in muscle and of L subunits (L4) in liver. A third subunit is found in platelets. Deficiency of the M subunit leads to glycogen storage disease type 7 (Tarui disease). It is characterised by muscle cramps and myoglobinuria on exertion. Shortened red cell viability may be a minor component of this disease. Evidence of haemolysis may be accompanied by mild erythrocytosis as a result of decreased production of 2,3-DPG.

Fructose diphosphate aldolase A deficiency

Fructose-1,6-diphosphate aldolase A (ALDOA) catalyses the conversion of fructose-1,6-diphosphate to Ga3P and dihydroxyacetone phosphate (DHAP). There are three aldolase isoenzymes in human tissues, A, B and C; A isoenzyme is expressed in red cells. ALDOA is produced in the developing embryo and also forms the bulk of the enzyme in muscle, where it may be as much as 5% of the total cellular protein. In the red cell, the reaction catalysed by the enzyme is virtually irreversible.

This enzyme deficiency is extremely rare, with only six patients described so far. The defect presents with anaemia, which may be associated with intellectual disability, dysmorphic features or myopathy. In one patient, rhabdomyolysis occurred.

Table 16.6 Main features of red cell enzyme deficiencies.

	Transmission	Gene	Chromosome	No. cases	No. mutations	Haematology	Other symptoms
Embden–Meyerhof pathway							
Hexokinase	AR	*HK1*	10q22.1	20 cases	5	CNSHA	
Glucose-phosphate isomerase	AR	*GPI*	19q13.11	>50 fam	31	CNSHA	Intellectual disability?
Phosphofructokinase	AR	*PFK-M* *PFK-L*	12q13.11 21q22.3	50–100 cases	23	Erythrocytosis Minimal haemolysis	Muscle disease, Tarui disease (Glycogenosis type VII)
Aldolase	AR	*ALDOA*	16p11.2	6 cases	4	CNSHA	Intellectual disability, Dysmorphism
Triosephosphate isomerase	AR	*TPI1*	12p13	50–100 cases	18	CNSHA	Neuromuscular disease Infections
Phosphoglycerate kinase	X-linked	*PGK1*	X13.3	40 cases	20	CNSHA	Neuromuscular disease
Pyruvate kinase	AR	*PKLR*	1q22	>500 fam	>200	CNSHA	
Rapoport-Luebering shunt							
Bisphosphoglycerate mutase	AR	*BPGM*	7q33	6 fam	3	Erythrocytosis, CNSHA	
Hexose-monophosphate shunt							
Glucose-6-phosphate Dehydrogenase	X-linked	*G6PD*	Xq28	>400×10^6 cases	>180	CNSHA–acute Favism	
Glucose-6-phosphate dehydrogenase (class I)	X-linked	*G6PD*	Xq28	> 50 fam	>60	CNSHA- chronic	
Glutathione metabolism							
Glutathione synthetase	AR	*GSS*	20q11.22	>50 fam	32	CNSHA	Metabolic acidosis (5-oxoprolinuria) Neurological symptoms Infections
Glutathione reductase	AR	*GSR*	8p21.1	2 fam	3	Induced oxidative HA, Favism, Neonatal jaundice	Cataract
γ-Glutamylcysteine synthetase (glutamate cysteine ligase)	AR	*GCLC* *GCLM*	6p12.1 1p21	12 fam	6	CNSHA, Oxidative HA	Metabolic acidosis (5-oxoprolinuria) Neurological symptoms
Glutathione peroxidase	AR	*GPX1*	3p21.3	1	0	Acute intravascular haemolysis?	
Nucleotide metabolism							
Adenosine deaminase (hyperactivity)	AD	*ADA*	20q13.12	3 fam	0	CNSHA	
Adenylate kinase	AR	*AK1*	9q34.11	12 fam	7	CNSHA	Motor impairment, Intellectual disability
Pyrimidine-5′ -nucleotidase	AR	*NT5C3A*	7p14.3	>60 fam	26	CNSHA	
NADH-cytochrome b5 reductase	AR	*CYB5R3*	22q13.2	50 cases	45	Methemoglobinemia	Neuromuscular disease, Intellectual disability

AD, autosomal dominant; AR, autosomal recessive; fam, families; CNSHA, congenital non-spherocytic haemolytic anaemia; HA, haemolytic anaemia.

Triose phosphate isomerase deficiency

Triose phosphate isomerase (TPI) catalyses the interconversion of DHAP and Ga3P. In red cells, all DHAP is converted to Ga3P, which is then metabolised down the glycolytic pathway, providing the ultimate two molecules of ATP for each molecule of glucose metabolised. The enzyme is a homodimer present in all tissues that is catalytically active only in its dimeric form. In tissues other than the red blood cell, DHAP is an important precursor for the biosynthesis of lipids. More than 50 cases of TPI have been reported. This produces a severe syndrome, present from birth, consisting of CNSHA, a progressive neurological disorder with spasticity and central nervous system (CNS) degeneration. Cardiac failure and sudden death due to arrhythmias are also features. Death occurs usually at about 5 years of age, likely because of the formation of toxic protein aggregates, induced by misfolded TPI. A number of point mutations have been identified that lead to the syndrome; the most predominant is Glu104Asp, linked by common haplotypes suggesting descent from a common ancestor.

Phosphoglycerate kinase deficiency

Phosphoglycerate kinase (PGK) catalyses the reversible conversion of 1,3-DPG to 3-phosphoglycerate, generating one molecule of ATP for each molecule of 1,3-DPG metabolised. The active enzyme in the red blood cell is encoded by the *PGK1* gene on the X chromosome. The enzyme is monomeric and is expressed in all tissues. PGK deficiency shows a wide clinical phenotype: it may present with chronic haemolysis, neurological impairment (intellectual disability and ataxia) and myopathy (exercise intolerance or muscle weakness). A few affected individuals suffer the full spectrum of symptoms, whereas some cases are described with myopathy without haemolysis.

Defence against oxidative stress: the production of reducing power

As with all cells, but perhaps more urgently, red blood cells need to be protected against the effects of free radicals, hydrogen peroxide and other highly oxidative material to maintain membrane integrity and functional activity. In addition, haemoglobin has to be maintained in its functional state, and the steady production of methaemoglobin reversed to deoxyhaemoglobin, which is able to combine reversibly with oxygen. The major generator of reducing power within the red cell is the pentose phosphate pathway, which generates reducing power in the form of NADPH from $NADP^+$ coupled to the oxidation of G6P to 6-phosphogluconate (6PG) and the subsequent oxidation of 6PG to ribose-5-phosphate, by the action of G6PD and 6-phosphogluconate dehydrogenase (Figure 16.14).

GSH is important for protecting cells from oxidative damage by these free radicals and the effects of infection, maintaining protein sulfydryl groups in the reduced state and maintaining membrane transport. GSH is provided by the glutathione cycle linked to the pentose phosphate pathway through the action of glutathione reductase (Figure 16.15).

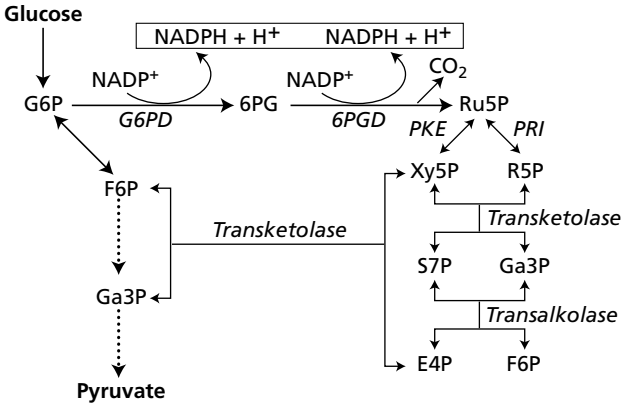

Figure 16.14 The pentose phosphate pathway. Substrates: G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; Ga3P, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; Xy5P, xylose-5-phosphate; S7P, septulose-7-phosphate; E4P, erythrose-4-phosphate. Enzymes: G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; PKE, phosphoketoepimerase; PRI, phosphoribose isomerase.

Oxidative stress that exceeds the reducing power of the red cell leads to intravascular haemolysis following denaturation of haemoglobin with precipitation as Heinz bodies and peroxidation of the red cell membrane. Methaemoglobinaemia may occur. The resulting clinical features – acute intravascular haemolysis, Heinz body haemolytic anaemia or methaemoglobinaemia – depend on the nature of the oxidative stress and the size of the imbalance between the stress and the redox potential.

Pentose phosphate pathway (hexose monophosphate shunt)

The pentose phosphate pathway, schematically shown in Figure 16.12 and in detail in Figure 16.14, is essential for the production of ribose and its incorporation into RNA. In red cells, its only function is the production of reducing power in the form of NADPH. The first step of the pathway, catalysed by G6PD, utilises G6P as substrate. G6P is also a substrate for glycolysis. About 10% of glucose is metabolised by the pentose phosphate pathway which is controlled by the availability of NAD^+ and the feedback inhibition of ATP. Under conditions of oxidative stress, the flux through the pentose phosphate pathway can be greatly increased. The products of the pathway re-enter the glycolytic pathway through F6P or Ga3P. The most frequent defect of the pentose phosphate pathway is G6PD deficiency. However, other enzymes can be important in acquired

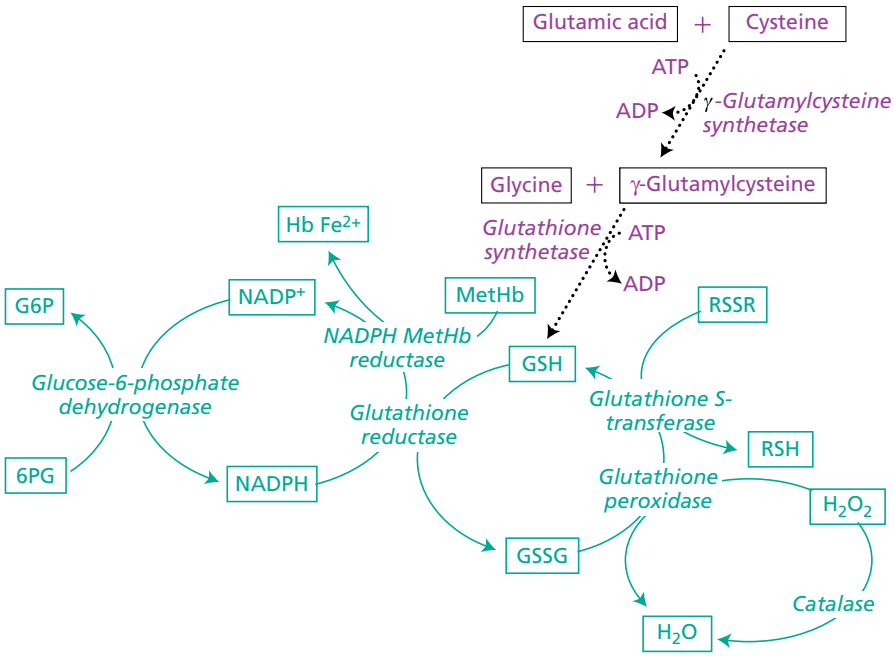

Figure 16.15 The glutathione (GSH) cycle and synthetic pathways. Redox control is exercised by the glutathione cycle linked to the NADPH of the pentose phosphate pathway by glutathione reductase.

disorders, some steps of the pathway being inhibited by drugs, including oestrogen/progesterone contraceptive pills.

Glucose-6-phosphate dehydrogenase deficiency

G6PD is the enzyme controlling flux through the availability of $NADP^+$. Conversion of G6P to 6PG is accompanied by the reduction of $NADP^+$ to NADPH (Figure 16.14); the oxidation of 6PG to ribose-5-phosphate produces a second molecule of NADPH. $NADP^+$ availability is determined by the glutathione cycle, which is linked to the pentose phosphate pathway through the activity of glutathione reductase (Figure 16.15).

The gene encoding for G6PD is located on the X chromosome at Xq28. The protein is assembled to produce an equilibrium of dimers and tetramers. Each monomer has an $NADP^+$-binding domain and a large domain with the active site between the two. The gene is highly conserved throughout evolution and is expressed in all cells, with an essential role in the production of RNA in nucleated cells. Complete inactivity of the enzyme in nucleated cells would be lethal. The clinical consequences of G6PD deficiency are virtually confined to the erythrocyte, with occasional evidence of leucocyte malfunction in some variants. Most mutations affect the stability of the enzyme so that its activity rapidly declines in the mature enucleated red cell as it ages. G6PD deficiency is the commonest genetically determined enzyme deficiency in the world, with an estimated 400 million people being affected. More than 180 different variants associated with deficient enzyme activity have been described and at least 35 of them are polymorphisms. The G6PD variants have been classified into five classes according to their activity relative to the wild-type G6PD type B (Table 16.7). In Africa, the variant G6PD type A^- is the predominant polymorphism, with equivalent activity to G6PD type B.

G6PD deficiency is widely found in people whose ethnic heritage relates to Africa, the Mediterranean basin, the Middle East, Southeast Asia and the Indian subcontinent. G6PD-A^- is common in Africa; the Mediterranean variant is common in Southern Italy, Sardinia and other Mediterranean regions and G6PD-Canton is common in Southern China. These variants are only the most common amongst many different mutations in these areas and throughout the rest of the affected world. This distribution of the deficiency equates with areas where *Plasmodium falciparum* malaria is common, and this is thought to be the evolutionary drive that has produced such widespread polymorphisms. It has subsequently been confirmed that G6PD deficiency does indeed protect against lethal *falciparum* malaria, particularly in childhood, and this protection, especially in hyperendemic areas, more than outweighs the haematological problems associated with deficiency.

There are four main syndromes associated with G6PD deficiency differing in their clinical presentations: neonatal jaundice, favism, chronic non-spherocytic haemolytic anaemia and drug-induced haemolytic anaemia. In all four, haemolysis is

Table 16.7 World Health Organisation classification of G6PD deficiency (1989).

Class	Enzyme activity (% normal)	Examples	Clinical effects
I	Severe (usually <2)	Santiago de Cuba (Gly447Arg)	CNSHA, acute exacerbations
II	<10	Mediterranean (Ser188Phe) Canton (Arg459Leu) Orissa (Ala44Gly)	Favism, acute intravascular haemolysis (drug-induced), neonatal jaundice
III	Moderate (>10, <60)	A– (Val68Met, Asn126Asp)	Acute intravascular haemolysis (drug-induced), neonatal jaundice
IV	100	B (wild type) A (Asn126Asp)	None None
V	150	//	None

CNSHA, congenital non-spherocytic haemolytic anaemia.

aggravated or promoted by exposure to oxidative stress through infection or ingestion of oxidative foods or drugs. Age always modifies the clinical effects, not always as might be expected.

The neonatal jaundice syndrome has been described in class I, II and III variants, favism mainly, though not exclusively, in class II, CNSHA in class I, and the drug-induced haemolytic anaemias mainly in class III. While G6PD deficiency is most common in males, the prevalence of the gene in many parts of the world means that female homozygotes are not uncommon and heterozygous females are often susceptible to oxidative stress because of the effects of X-inactivation and marked lyonisation resulting in a significant population of G6PD-deficient red cells.

Neonatal jaundice and G6PD deficiency in infancy

Neonatal jaundice is a severe manifestation of G6PD deficiency and is a major source of potential morbidity from kernicterus. Most common variants have been associated with the syndrome, including the A^- and Mediterranean variants. In parts of the world where G6PD is common, it is the most prevalent cause of neonatal jaundice. The jaundice probably starts *in utero* during the perinatal period, but the clinical problem only becomes apparent on about the second or third day after birth. Phototherapy or exchange transfusion may be required to prevent neurological sequelae. Anaemia is not a feature, and it is thought that jaundice is a manifestation of liver enzyme deficiency coupled perhaps to the physiological underdevelopment of neonatal liver function or the coinheritance of Gilbert's syndrome. Acute haemolytic crises may occur in infants, usually through exposure to oxidative stress, including nitrites or nitrates in water or the ingestion of fava beans by the mother, but in some cases of severe acute haemolysis, even fatal, no cause has been obvious.

Favism

Favism is the term given to the G6PD syndrome where acute intravascular haemolysis may be precipitated by exposure to the broad bean *Vicia fava*, ingested in any form, usually about 24 hours after the exposure (Table 16.9). The offending agent is divicine, or its aglycone isouramil, which can produce free oxygen radicals on autoxidation. Divicine is not present in peas or beans of other types, which may be eaten without effect. The amount of haemolysis is dose-related, which may explain the marked variation in susceptibility not only in different subjects, but also in the same individual at different times. In children, acute haemolysis, sometimes life-threatening, is common, but renal failure is uncommon, although there may be systemic symptoms of fever and loin pain. Acute kidney injury occurs more often in adults, possibly because of comorbidity. Favism is usual in class II variants, for example Mediterranean and Canton, but may occur in others, including the African A^- variant. Acute haemolysis after eating fava beans has also been described in glutathione reductase deficiency. Some other compounds with which affected individuals may come in contact can cause acute haemolysis, including topical henna and some of the pulses used to make local sweetmeats.

Between attacks of favism or exposure to oxidising substances, the blood count is normal with no evidence of haemolysis. The cooccurrence of infection, which promotes the formation of hydrogen peroxide following the oxygen burst in neutrophils and macrophages with ingestion of oxidising substances, even mild ones such as chloramphenicol, may promote haemolysis, even though the drug on its own does not.

Chronic non-spherocytic haemolytic anaemia

Sporadic cases of CNSHA are found with underlying G6PD deficiency. Many of the mutations that cause CNSHA occur on exon 10 and affect the formation of dimers or tetramers.

Table 16.8 Characteristic features of haemolytic attack in G6PD deficiency.

Phase	Clinical	Laboratory
Acute	Abrupt onset	
	Malaise, prostration	
	Pallor	Anaemia, Heinz bodies, reticulocytosis, G6PD deficient
	(Abdominal pain)	
	Fever	Leucocytosis
	Dark urine	Haemoglobinuria, haptoglobin absent
	Haemoglobinaemia	
	Methaemalbuminaemia	
	Jaundice	Hyperbilirubinaemia
	(Renal failure)	↑ Urea, ↑ creatinine
Recovery	Gradual but rapid cessation of haemolysis	Reticulocytes peak days 5–8
	Urine clears in few days	G6PD increases (rarely to normal range)
	Jaundice clears in 1–2 weeks	

The haemolysis is extravascular, although additional oxidative stress may provoke an acute intravascular episode.

Drug-induced acute haemolysis

The introduction of primaquine and its derivative pamaquine as antimalarials to replace quinine during the Pacific phase of the Second World War and the later Korean War revealed that a proportion of men exposed, particularly in the black population, suffered from severe acute intravascular haemolysis. Studies by Carson and others from the University of Chicago, working at the Statesville Penitentiary Malaria Project, identified the problem as G6PD deficiency and identified that young red cells and reticulocytes had sufficient activity to withstand the oxidative stress so that haemolysis lessened as the reticulocyte level rose. It became apparent that many other drugs could also produce haemolysis. The common African A^- variant is the main example of class III mutations producing this type of haemolysis.

The haemolysis is dose-related and may be self-limiting. Although it is important to recognise which drugs are likely to produce haemolysis (Table 16.9), it is also important to realise that the disease for which the drugs may be needed, for example *falciparum* malaria, may be fatal, and thus the concern for haemolysis is a lesser problem.

Laboratory diagnosis

Acute intravascular haemolysis raises the suspicion of G6PD deficiency. Red cell morphology shows contracted haemoglobin in 'ghost' membranes (Figure 16.16). Haemoglobinuria may be gross, producing almost black urine without red cells in the centrifuge deposit. Several screening tests have been devised to identify G6PD deficiency in red blood cells. These tests can reliably distinguish between deficient and non-deficient individuals but are not reliably quantitative. The gold standard is the quantitative assay of G6PD activity. Since G6PD activity is red blood cell age-dependent the measurement of enzyme activity might yield false-normal results in the presence of reticulocytosis, (i.e. during haemolytic attack). The diagnosis of heterozygous females can be especially difficult; in extreme cases, it can only be done by family studies. However, the probability of clinically significant haemolysis in a heterozygote roughly correlates with the proportion of G6PD-deficient red cells in her blood. Therefore, if a normal level of G6PD activity is found in a heterozygote, she is unlikely to be at risk of G6PD-related haemolysis. DNA analysis is the most effective way of identifying heterozygotes, in particular in regions where G6PD has a high prevalence and the main variants are known.

Management

Management is mostly dictated by the symptoms and signs in patients, although education in the avoidance of oxidising substances is important (Table 16.8). In many populations, the condition is well known and the need for avoidance is recognised. Neonatal jaundice may need urgent therapy to prevent neurological damage. Extreme hyperbilirubinaemia can be prevented by administration of Sn-mesoporphyrin if the diagnosis is known at birth. Acute intravascular haemolysis may require transfusion, but the anaemia is often self-limiting. High fluid intake should be encouraged to prevent renal damage. CNSHA may be severe enough to warrant active treatment and splenectomy may be helpful.

Glutathione

GSH is the major intracellular thiol in aerobic cells and is equally important in the red cell. It has a number of critical functions: protecting cells against oxidative damage, participation in

Table 16.9 Drugs to be avoided in G6PD deficiency.

Category of drug	Predictable haemolysis	Possible haemolysis
Antimalarials	Dapsone Primaquine Methylene blue	Chloroquine Quinine
Analgesics/Antipyretic	Phenazopyridine	Aspirin (high doses) Paracetamol (Acetaminophen)
Antibacterials	Cotrimoxazole Sulfadiazine Quinolones (including nalidixic acid, ciprofloxacin and ofloxacin) Nitrofurantoin	Sulfasalazine
Other	Rasburicase Toluidine blue	Chloramphenicol Isoniazid Ascorbic acid Glibenclamide Vitamin K Isosorbide Dinitrate

Similar tables have also been published previously (e.g., Betke *et al.* 1967; Cappellini and Fiorelli, 2008; Luzzatto, 2012). The *Predictable haemolysis* column includes all seven drugs listed in a recent evidence-based review (Youngster *et al.* 2010). We consider that even a single case of AHA must be taken seriously if the clinical picture is well documented and if AHA is unlikely to have had another cause (e.g. infection): therefore, in addition to those seven drugs, we have retained in this column cotrimoxazole (of the two chemicals present in cotrimoxazole, sulfamethoxazole rather than trimethoprim is likely to be the culprit); sulfadiazine; and quinolones. The *Possible haemolysis* column is based on older literature (see for instance Burka *et al.* 1966; WHO Working Group, 1989). As examples of the causal role of some of these drugs in causing AHA in G6PD-deficient persons see Sicard *et al.* (1978) for chloroquine, Meloni *et al.* (1989) for aspirin, Mehta *et al.* (1990) for ascorbic acid, Meloni and Meloni (1996) for glibenclamide
(*Source:* Luzzatto and Seneca, 2014. Reproduced with permission of Wiley.)

detoxification of foreign compounds, maintenance of protein sulfhydryl groups in a reduced state and possibly transport of amino acids. In red cells, its main function is as an antioxidant. GSH is synthesised from glutamate, cysteine and glycine by the link reactions of two enzymes, γ-glutamylcysteine synthetase and glutathione synthetase (Figure 16.15). GSH exerts its function in preserving thiol groups and reducing hydrogen peroxide and free oxygen radicals through reactions catalysed by glutathione-*S*-transferase and glutathione peroxidase, respectively. Oxidised glutathione (GSSG) is reduced to GSH by the action of glutathione reductase, the hydrogen donor being NADPH. Failure to maintain the GSH level leads to chronic haemolytic anaemia and increased susceptibility to oxidative stress.

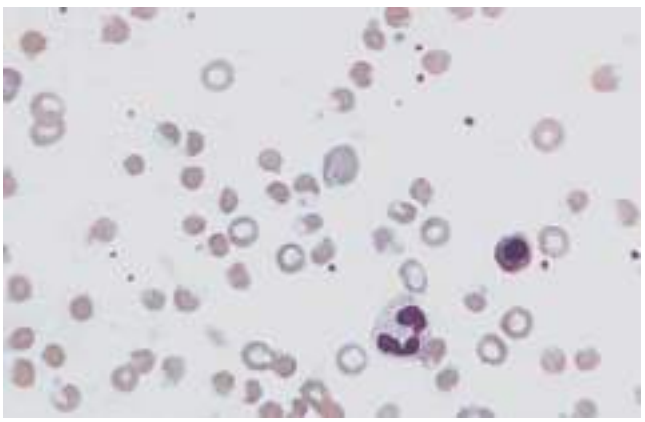

Figure 16.16 G6PD deficiency. Peripheral blood film following acute oxidant drug-induced haemolysis shows an erythroblast and damaged red cells, including 'blister' and 'bite' cells.

Complete loss of GSH synthesis is probably lethal. Severe deficiency leads to 5-oxoprolinuria, metabolic acidosis and intellectual disability. A milder deficiency limited to red cells is associated with haemolytic anaemia aggravated by oxidative stress. Low levels of GSH caused by γ-glutamylcysteine synthetase or glutathione synthetase deficiency, with CNSHA, have been described (Table 16.5). Haemolytic anaemia due to deficiency of glutathione reductase has also been reported.

Nucleotide metabolism

Adenosine nucleotides, ATP (85–90%), ADP (10–12%) and AMP (1–3%) comprise the main nucleotide pool in mature

red cells. The cell has no mechanism for making nucleotides once the RNA of the reticulocytes has been degraded, and it has an effective salvage mechanism for maintenance of the adenine pool, with the enzymes adenosine deaminase (ADA) and adenylate kinase involved in regulation. Deficiency of the enzymes in the salvage pathway does not usually lead to haemolysis. ADA deficiency is associated with severe combined immunodeficiency and excess activity is found in Diamond–Blackfan anaemia. During maturation of reticulocytes the RNA is broken down to pyrimidine and purine nucleotides, which are dephosphorylated to nucleosides that can diffuse out of the cell. The purine nucleotides enter the salvage pathway. Deficiency of pyrimidine 5′ -nucleotidase leads to accumulation of pyrimidine nucleotides that interferes with the adenine nucleotide pool, producing haemolysis (Table 16.5).

Pyrimidine 5′-nucleotidase

Pyrimidine 5′-nucleotidase (P5N), catalyses the dephosphorylation of the pyrimidine 5′-monophosphates uridine monophosphate (UMP) and cytidine monophosphate (CMP) to corresponding nucleosides (Figure 16.17). In red cells, there are two isoforms, type 1 (P5N1), which has a high affinity for UMP and CMP, and type 2 (P5N2), which hydrolyses deoxypyrimidine nucleotide monophosphates. P5N1 deficiency leads to haemolytic anaemia. The gene (*NT5C3*, also named P5′ N1), codifies for the two isoforms by exon 2 alternative splicing. The enzyme is strongly inhibited by lead.

P5N1 deficiency is not uncommon, it is probably the third most common enzyme deficiency causing CNSHA. It is characterised by marked basophilic stippling in the red cells (Figure 16.18), similar to that seen in lead poisoning, and accumulation of high concentrations of pyrimidine nucleotides. The haemolysis is usually mild to moderate, although more severe cases have been reported. Splenectomy usually results in stabilisation of the haemoglobin to higher levels (median increase 32 g/L).

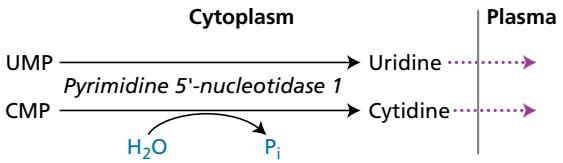

Figure 16.17 Pyrimidine nucleotide catabolism in the reticulocyte. Pyrimidine 5′ -nucleotidase converts the nucleotides to monophosphates, which diffuse out of the cell.

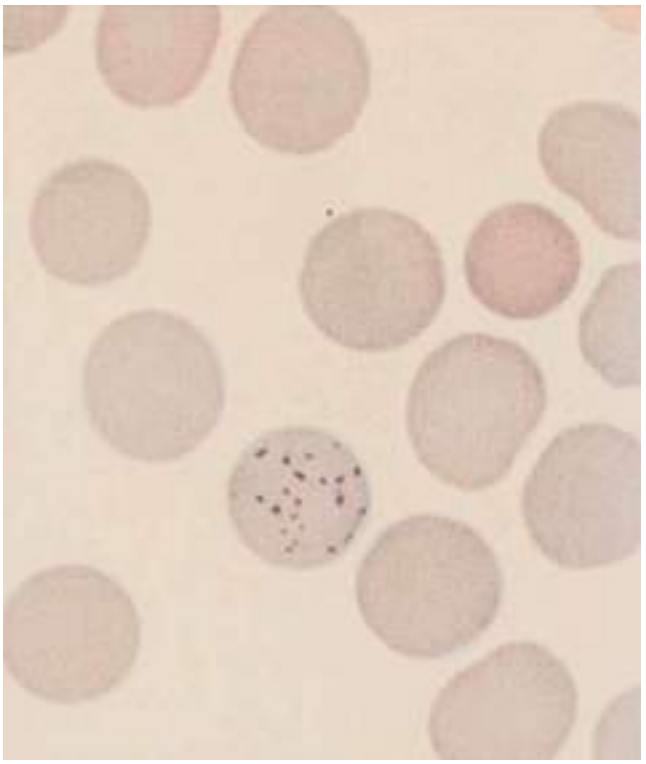

Figure 16.18 Peripheral blood film in pyrimidine 5′ -nucleotidase deficiency showing basophilic stippling.

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CHAPTER 17

Acquired haemolytic anaemias

17

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Introduction

Haemolytic anaemias result from significant shortening of the red cell life span and can result from either acquired or inherited causes. The acquired haemolytic anaemias are divided into two main categories based on the mechanism by which premature destruction of red blood cells is produced. In the immune haemolytic anaemias, antibodies are the main agents of destruction, while the non-immune acquired haemolytic anaemias result from diverse causes and mechanisms.

Immune haemolytic anaemias

Antibody-mediated haemolysis is an important cause of acquired haemolytic anaemia. These antibodies may be autoantibodies produced by a patient's immune system and directed against epitopes of their own red cell antigens, or they may more rarely be alloantibodies. Alloantibodies may be produced by the patient and directed against antigens either introduced by blood transfusion of donor red cells or secondarily acquired as in drug-induced haemolysis. Alloantibodies directed against the patient's red cell antigens might also be introduced from outside the patient, as occurs from the mother in haemolytic disease of the fetus and newborn. A simple classification of immune haemolytic anaemias is given in Table 17.1.

Immune haemolytic anaemias are distinguished from the non-immune by detecting antibody on the surface of red cells using the direct antiglobulin test (DAT).

Autoimmune haemolytic anaemia

Autoimmune haemolytic anaemia (AIHA) is a rare disease with an incidence of 1–2 cases per 100,000 per year, it has a highly variable clinical course ranging from mild disease to severe life-threatening haemolysis. About half of AIHA cases are classed as primary when no underlying or associated disorder is found, the rest are classed as secondary, related to an underlying cause that may be malignant (e.g. chronic lymphocytic leukaemia, CLL), autoimmune (e.g. systemic lupus erythematosus SLE), infective (including SARS-CoV2) or drug-induced (e.g. cephalosporins). AIHA can also occur in the context of solid organ or haematopoietic stem cell transplantation.

AIHA is a heterogeneous disorder which can be serologically divided into warm AIHA (wAIHA), accounting for two-thirds of cases; cold agglutinin disease (CAD), the second most common cause, affecting patients commonly in their 7th and 8th decades of life; mixed AIHA; paroxysmal cold haemoglobinuria (PCH), a rare condition that typically affects children; and atypical AIHA, a recently defined subgroup of DAT negative AIHA which may be driven by IgA or warm IgM.

Pathogenesis of AIHA

The pathogenesis of AIHA is complex and not yet fully elucidated, but as with other autoimmune disorders, its development is associated with the presence of autoreactive T and B cells as well as the dysregulation of self-tolerance. T helper type 17 cells (Th17) cells are engaged in autoimmunity and the development of inflammation, whereas CD4+ and CD25+ T regulatory cells (Tregs) inhibit the development of autoimmunity and contribute to self-tolerance by suppressing

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 17.1 Classification of immune haemolytic anaemias.

Antigen type	Antibody	Diseases	Associations
Autoimmune	Warm antibody	Primary Secondary	Idiopathic Autoimmune diseases (ITP, SLE, Rheumatoid arthritis) Lymphoproliferative disorders Infections (EBV, SARS-CoV2) Ovarian carcinoma and some other cancers
	Cold antibody	Cold agglutinin disease (CAD) Cold antibody syndromes	Drugs Infections (*M. pneumoniae*), lymphoproliferative disorders
	Donath–Landsteiner antibody	Paroxysmal cold haemoglobinuria (PCH)	Post viral, syphilis
Alloimmune	Induced by red cell antigens	Haemolytic transfusion reactions Haemolytic disease of the fetus and newborn (HDFN) Post haematopoietic stem cell transplant	
	Drug dependent	Antibody/macrophage-mediated Antibody/complement-mediated Membrane modification Autoimmune	

EBV, Epstein–Barr virus; ITP, immune thrombocytopenia; SLE, systemic lupus erythematosus.

auto-reactive T cells. In AIHA, there is an imbalance due to an increase in the Th17 compartment.

There is also a breach of the normal interaction between several other components of the immune system including antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by macrophages, cytotoxic T cells, NK cells and autoantibodies. Interactions between cytokines, B cells and reactive oxygen species (ROS) have also been implicated, as have reticulocytes which some models have shown to be a target for pathogenic autoantibodies. There is gathering evidence on the role of shear stress in the induction and progression of AIHA, as well as genetic pre-disposition; next generation sequencing has revealed recurrent *KMT2D* mutations in CAD patients for example.

The risk of acquiring AIHA increases significantly with age, and this age-related effect is likely contributed to by immunosenescence (age-related decline of the immune system) or an age-related accumulation of epigenetic abnormalities in the haematopoetic stem cell.

Characteristics and specificity of red cell autoantibodies

Autoantibodies typically show reactivity against a number of antigens and are not as specific as alloantibodies. In AIHA, the antibody type and its thermal characteristics influence both the site and intensity of red cell destruction. The direct antiglobulin test (DAT) is the cornerstone of diagnosis in AIHA and is used to evaluate the anti-red cell antibodies using routinely available multi- and mono-specific anti-human globulin reagents which detect IgG, IgM, IgA and the complement components C3c and C3d. The most frequent patterns detected by DAT on the red cell surface in AIHA are IgG alone, IgG and complement, and complement alone. True DAT-negative AIHA is rare: some initial apparently DAT-negative cases will be due to low-affinity antibodies which can be overcome by low ionic strength solution or cold washing. Newer, more sensitive assay methods including flow cytometry may be useful. Despite extensive work-up a small fraction of AIHA cases remain DAT negative, but this is a diagnosis that can only be made after excluding many other causes of haemolysis.

Warm-acting antibodies

WAIHA autoantibodies bind to red cells more strongly at 37 °C and have decreased affinity at lower temperatures; they are polyclonal and IgG antibodies predominate. Complement activation is seen when the antibody titre is markedly raised or when IgG1 or IgG3 subclasses are involved. These antibodies may be detected in the serum by the indirect antiglobulin test (IAT) in about 50–60% of patients, rising to more than 90% of cases when the red cell membrane of the reagent cells is modified with papain or another proteolytic enzyme. In most cases of warm-type AIHA, the antibody detected in the patient's serum is pan-reacting with all cells in a routine group O panel. Where specificity of the antibody can be determined, it is most commonly in the Rh blood group complex, with 10–15% showing specificity for either anti-e, anti-D or anti-c.

A greater proportion shows specificity by reacting with all cells except –/– Rh^{null} cells. Other rare specificities against high-frequency antigens include anti-En^a, anti-Wr^b or anti-U.

Cold-acting antibodies

Cold-acting antibodies are predominantly IgM and are most actively bound to antigen in the cold (4 °C), with little affinity at physiologic temperatures. These antibodies act both as agglutinins and lysins *in vitro*; the two functions may have different thermal ranges. The clinical significance of the antibody is not related to the titre of the cold antibody, but depends on the thermal range of its activity and how the antibody binds to red cells at or near 37 °C. IgM antibodies active against the I (auto) antigen at 4 °C can be a normal finding and, although they have a wide range of thermal activity, they produce no clinical disturbance above 32 °C. *In vitro*, the IgM antibodies elute off the red cell membrane, leaving bound complement to be detected by anti-C3d in the DAT.

According to a recent international AIHA consensus document, primary cold agglutinin disease (CAD) is defined as an AIHA characterised by a monospecific DAT strongly positive for complement fragment C3d, usually negative for IgG (although it may be weakly positive in up to 20% of cases). There is usually an underlying indolent B-cell lymphoproliferative disorder that produces the cold agglutinin (CA) which initiates the haemolysis. CA are usually a monoclonal IgM (κ) with an optimum temperature of reaction at 4 °C (thermal range 4–34 °C), which determines their pathogenicity. They bind to red cells in the cooler peripheral circulation, which can result in agglutination of the red cells leading to acrocyanosis or Raynaud's disease in some patients. After binding, the IgM-antigen complex initiates the classical complement pathway resulting in opsonisation of the red cell by C3b. Once warmed in the central circulation the IgM detaches from the red cells allowing agglutinated cells to separate, but C3b remains bound to the red cell membrane. C3b opsonised red cells are prone to phagocytosis; on the red cells that survive this process, the surface C3b is further cleaved to C3d which can be detected by DAT.

Secondary cold agglutinin syndrome (CAS) is a similar to CAD, but the associated clinical disease may be an aggressive lymphoma or an infection with *Mycoplasma pneumoniae* or Epstein Barr virus. It may also occur secondary to an autoimmune disorder or malignancy. The cold agglutinin associated with infection and autoimmune diagnoses tends to be a polyclonal IgM, however, CAS cases associated with an overt (clinical or radiological) lymphoma will be monoclonal. Polyclonal CA may also be detected in low titres in a proportion of the adult population without any haemolysis or clinical symptoms.

These cold agglutinins (IgM antibodies) tend to have specificity for the I/i blood group system. I predominates from 18 months age onwards hence most patients' cold agglutinins are specific for the I antigen. Very occasionally, there is specificity for other antigens including anti-Pr, anti-P, anti-M or anti-N.

The antibodies in a subtype of cold AIHA, paroxysmal cold haemoglobinuria (PCH), are of IgG type and bind to antigen below 20 °C. When the temperature is raised to 37 °C in the presence of complement, lysis occurs. This biphasic reaction is the basis of the Donath–Landsteiner reaction. In PCH, the specificity of the autoantibody is anti-P.

Complement activation

Antibody binding to two adjacent sites on the red cell membrane is required to activate the C1 complement component by the classical pathway. IgM molecules are pentameric and a single molecule can bind adjacent sites whilst IgG molecules can only activate complement if they form a 'doublet'. IgG1, IgG2 and IgG3 can activate complement, whereas IgG4 and IgA do not. In AIHA, complement activation usually stops at the C3 stage where C3b is bound to the membrane and further proteolysed to form the inactive component C3d, which is detected by the appropriate DAT. Complement activation beyond the C3 stage may lead to the formation of the membrane attack complex resulting in intravascular haemolysis. AIHA due to IgG2 alone is very rare and that due to IgG4 or IgA alone is uncommon. It is interesting that in the rare case of IgA AIHA, complement does become activated on the cell surface, although the mechanism is obscure as IgA does not itself fix complement. In general, when there is more than one class or subclass of antibody on the cell surface, the haemolysis is more intense and may be intravascular.

Mechanisms for immune red cell destruction

There are two main mechanisms of red cell destruction *in vivo:* first, cell-mediated, predominantly extravascular haemolysis, and second complement-mediated intravascular haemolysis. Haemolysis in AIHA is mainly extravascular: the red cells are commonly coated with IgG only and preferentially destroyed in the spleen. In the cases where they are coated with a combination of IgG and complement or complement alone, the main site of destruction is the liver, however, intravascular haemolysis may also occur. The site and severity of red cell destruction depend on the structural and functional characteristics of the antibody and the efficiency of the mechanism of destruction, while the degree of anaemia depends on the rate of destruction versus the capacity of the bone marrow to compensate.

Cell-mediated immune destruction of antibody-coated red cells is carried out by macrophages of the reticuloendothelial system within the spleen, liver and bone marrow. These cells have cell-surface receptors for the Fc portion of IgG and the antigenic determinants present on activated C3. Macrophages have Fc receptors for IgG1 and IgG3 molecules, but not for IgG2, IgG4, IgM or IgA. Hence only IgG-coated red cells are

destroyed in this way. It is the main method of haemolysis in warm AIHA where 70–75% of autoantibodies are IgG. Phagocytosis and antibody-dependent cell-mediated cytotoxicity are the major Fc receptor-dependent modes of antibody-coated cell destruction.

Role of the spleen

The splenic vasculature is adapted to provide an efficient filter for particles such as effete red cells, bacteria and immune complexes. As blood passes through the central arteries towards the red pulp, the branches of these arteries have a plasma-skimming effect that raises the haematocrit of the blood as it passes towards the splenic cords. There, red cells come into close contact with splenic macrophages. The low plasma content and the relative lack of free plasma IgG molecules allow red-cell-bound IgG to interact preferentially with macrophage Fc receptors, leading to phagocytosis of coated red cells. When phagocytosis is partial, so that only portions of the cell membrane are removed, the remaining circulating red cell becomes spherocytic, although the somewhat rigid spherocytes may themselves be trapped in the splenic sinusoids and destroyed. The spleen is therefore the major site of red cell destruction when IgG alone is the main Fc-binding protein on the red cell surface.

Role of the liver

Macrophages that are present in the liver sinusoids (Kupffer cells) express Fc receptors on their surface. Blood flow through the sinusoids is rapid and compared with the spleen and there is no plasma-skimming effect so IgG-coated red cells are not preferentially destroyed in this situation, instead, there is competition for the Fc receptors from circulating IgG and red cell destruction is more dependent on cells being coated with C3.

Two types of C3 receptor have been identified in macrophages: CR1 and CR3. CR1 is specific for an antigenic site in the C3c region of activated C3b, and its breakdown product iC3b which is the only ligand for CR3. Immune adherence of C3b-coated red cells to macrophages occurs mainly through CR1, whereas CR3 binding triggers phagocytosis. The largest concentration of C3b-binding macrophages is found in liver sinusoids, and hence the liver is the major site for trapping and phagocytosing C3b-coated red cells. There is no competition for complement receptor sites from non-activated C3 in the plasma. When markedly enlarged, the spleen also becomes an important site of cell destruction.

Complement-mediated intravascular haemolysis

Complement-mediated intravascular haemolysis is a minor mechanism for red cell destruction in most patients with AIHA, however in a small proportion of patients when more than one class or subclass of immunoglobulin is present on the red cell surface, such a mechanism may predominate and produce severe intravascular haemolysis.

In warm AIHA, complement-induced intravascular haemolysis has been reported with IgA-coated red cells, although the mechanism is obscure, as IgA does not itself fix complement.

In cold AIHA syndromes, intravascular haemolysis may be precipitated by exposure to cold. In such cases, lytic as well as agglutinating antibodies with a high thermal range may be demonstrated *in vitro*, a pattern that may be found in cold AIHA associated with *Mycoplasma pneumoniae* infection. Acute intravascular haemolysis is the usual presentation in PCH, where the antibody is IgG in type.

Other factors influencing red cell destruction and production

The bone marrow's function and capacity to increase erythropoiesis following significant red cell destruction may be impaired by autoantibodies that bind to and destroy reticulocytes and erythroblasts as well as the mature red blood cells. Red cell production may also be reduced by acute folate deficiency secondary to increased demand. In lymphoproliferative disorders, red cell production can be impaired by bone marrow infiltration.

Reticuloendothelial function plays an important role because the severity of cellular immune red cell destruction depends largely on macrophage function. Reticuloendothelial function may be reduced in SLE by the clearance of immune complexes, a process known as reticuloendothelial blockade. In methyldopa-induced AIHA, the drug has been shown to reduce reticuloendothelial clearance of IgG-coated red cells, a possible explanation for the fact that many patients with a strongly positive DAT due to methyldopa have little or no haemolysis.

Hypocomplementaemia or a relative complement deficiency may result from continuous complement activation such as occurs in patients with chronic CHAD. Hypocomplementaemia is common in SLE and there is a strong association between SLE and the occurrence of null alleles for the C2 and C4 genes, which causes a genetically determined complement deficiency.

Clinical features of warm AIHA

Warm-type AIHA affects all age groups and accounts for up to 70% of AIHA cases. The haemolysis is caused by high-affinity IgG antibodies produced by reactive polyclonal B cells. An underlying or associated disorder can be identified in 50–70% of cases and there is a preponderance of female patients in both idiopathic and secondary AIHA.

The clinical presentation is variable and depends on the speed with which anaemia develops, the capacity of the bone marrow to compensate, and the effects of any associated disease. Typically, the onset is insidious, with gradual awareness

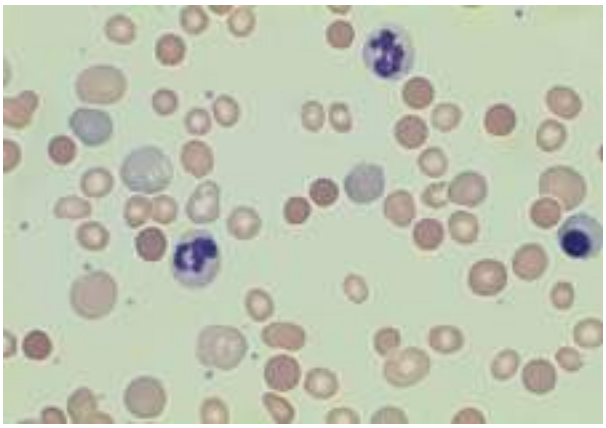

Figure 17.1 Warm autoimmune haemolytic anaemia. Blood film showing spherocytosis (arrows), polychromasia and a nucleated red blood cell (×40).

of symptoms of anaemia or observation of pallor or mild icterus by friends or relatives. Occasionally, the onset is acute, with rapidly developing anaemia and, in older patients, the risk of heart failure. Rarely, severe fulminant haemolysis may occur, resulting in life-threatening anaemia. A moderate increase in unconjugated serum bilirubin and excess urinary urobilinogen occurs as a result of extravascular haemolysis. Where intravascular haemolysis occurs, patients pass very dark urine caused by haemoglobinuria. More marked icterus (bilirubin >90 μmol/L) suggests coexisting liver disease or biliary tract obstruction due to pigment gallstones or biliary sludge. Mild splenomegaly is common, rarely more than 2–3 cm below the costal margin; marked splenomegaly suggests the possibility of an underlying lymphoproliferative disorder.

Anaemia with marked reticulocytosis is present and the peripheral blood film is characterised by polychromasia, spherocytes (Figure 17.1), circulating nucleated red cells and, in some cases, red cell agglutination. Rarely, there may be reticulocytopenia associated with a positive DAT. There is an increase in lactate dehydrogenase (LDH) due to red cell lysis, but other liver function tests are normal unless there is associated liver or biliary tract disease. The DAT is positive, commonly with IgG alone (mainly IgG1 and IgG3 subclasses) or with IgG and complement (the latter pattern commonly seen in SLE). Autoantibody in the serum may show specificity within the Rh system (e.g. auto anti-e), but in most cases is pan-reactive with all red blood cells. The autoantibody reacts at 37 °C. In very rare cases, the amount of antibody remaining on the red cell surface is insufficient to be detected by the conventional DAT.

Idiopathic warm AIHA

Idiopathic warm AIHA with no underlying cause or associated disorder accounts for approximately 30% of patients with a DAT-positive haemolytic anaemia. It may occur at any age; peak incidence occurs in infancy and early childhood with a second rise during the third decade and the majority of cases occurring after the fifth decade. In infancy, the onset is often acute, typically precipitated by infection and transient. However, anaemia may be profound and difficult to control. There is a male preponderance in the childhood setting in contrast to females in adult cases. IgG antibodies can be transferred from an affected mother across the placenta, producing haemolysis in the newborn. A careful drug history should be taken in all cases to exclude haemolysis induced by drugs or by chemical exposure. In girls, AIHA may precede clinical or immunological evidence of SLE which should not be excluded on account of initial negative serology. Clinical presentation may vary from gradual onset of symptomatic anaemia to an acute, fulminating and life-threatening haemolytic process. Systemic symptoms include pallor, jaundice and mild splenomegaly (1.5–5 times normal size). Marked splenomegaly suggests a secondary cause.

Evans syndrome

Evans syndrome is defined as the combination of AIHA and immune thrombocytopenia (ITP) in the absence of any underlying disorder. The onset of thrombocytopenia may coincide with haemolysis or may arise separately. The platelet and red cell antibodies are distinct and do not cross-react. Rarely, episodes of immune neutropenia or pancytopenia have also been described in association with a positive DAT. The diagnosis is important because there appears to be a higher incidence in these cases of underlying conditions including immunodeficiency or autoimmune lymphoproliferative disease (ALPS) in children and of SLE or T-cell lymphoma in adults. Typically, the condition runs a chronic course characterised by relapses and remissions.

Warm AIHA associated with other autoimmune diseases

This is well described in patients with SLE, especially in young women, and in other autoimmune or presumed autoimmune diseases, notably rheumatoid arthritis, Sjögren syndrome and ulcerative colitis. Warm AIHA is also part of the spectrum of autoimmune diseases associated with agammaglobulinaemia. Autoantibodies are usually IgG, and both IgG and C3d are found on the red cell surface. Occasionally, the DAT may be positive because of immune complexes adsorbed onto the red cell surface. The spleen is important for clearing such coated cells and splenectomy should be avoided if possible. Otherwise, treatment is as for idiopathic AIHA (see below).

Lymphoproliferative diseases

Lymphoproliferative diseases, including chronic lymphocytic leukaemia (CLL) and other forms of low-grade B-cell non-Hodgkin lymphoma are well described in association with cases of warm AIHA. The AIHA may precede the diagnosis of lymphoma by

months or years, may occur simultaneously with onset of the lymphoma, or occur afterwards. Antibody response is thought to be due to immune dysregulation rather than direct production by the malignant clone. Antibodies are polyclonal and have no distinct pattern of type or specificity. Refractory autoimmune cytopenias are also well described following the use of therapeutic agents for lymphoma including fludarabine and alemtuzumab, probably the result of their powerful effect on the immune system.

Drug-related warm AIHA

Antibody-induced haemolytic anaemia caused by drugs is rare with an estimated incidence of approximately 1–4 cases per million individuals per year, but it can result in an acute, brisk and potentially life-threatening haemolysis. More than 130 drugs have been implicated, most commonly antibiotics, especially the second- and third-generation cephalosporins, and platinum-based chemotherapies.

Drugs typically have a molecular weight that is too low to be immunogenic (hapten), unless they are conjugated with a larger carrier molecule such as a protein, which then allows them to elicit an immune response. Haemolysis results from antibodies directed against self-antigens induced by a drug and these divide into two broad mechanisms. The first is via drug-dependent antibodies that activate the immune response only while the drug is present; the second is due to drug-independent antibodies, which create an autoimmune response in the absence of the drug. The first mechanism is more common. How these drugs induce autoantibody formation has not been elucidated and a variety of mechanisms have been postulated; however, the same drug at different doses or repeated usage may activate different mechanisms and there are recent suggestions that membrane modification may underlie most of the mechanisms.

Drug adsorption and extravascular haemolysis:

Penicillin is the prototype drug for this mechanism, although other penicillin derivatives and cephalosporins have also been implicated. These drugs form hapten–carrier complexes with plasma proteins enhancing drug-specific antibody production such that an estimated 90% of individuals receiving penicillin will produce clinically insignificant IgM anti-penicillin antibodies. When high-dose intravenous penicillin is administered, the drug is adsorbed onto the red cell surface and becomes non-specifically attached to cell-surface proteins. A minority of patients on high-dose intravenous penicillin therapy (>l million units daily) develop high-titre IgG anti-penicillin antibodies that attach to the drug bound to the red cell surface causing predominantly extravascular haemolysis. Typically, mild to moderate haemolysis occurs, but if unrecognised and large doses of drug are continued in the presence of increasing antibody levels, complement fixation and acute intravascular haemolysis may occur. The DAT, due to IgG on the red cell surface, becomes positive some weeks following treatment. When the drug is stopped, haemolysis stops and the DAT rapidly becomes negative. Antibody in the patient's serum or eluate from the red cells will react with normal red cells only in the presence of the drug. The clinical and serological features are shown in Table 17.2.

Immune complex and complement-activated acute intravascular haemolysis:

Several drugs have been reported to cause immune haemolytic anaemia by this mechanism. Most frequently reported are rifampicin, phenacetin, quinine and, more recently, intravenous cephalosporins and diclofenac (see below). Hapten–carrier complexes are formed between these drugs and plasma proteins, leading to the production of drug-specific antibodies. Once drug antibodies are present, reintroduction of the drug causes

Table 17.2 Drug-induced immune haemolytic anaemias: clinical and serological features.

	Drug adsorption mechanism	Immune complex mechanism	Autoimmune mechanism	Membrane modification mechanism
Examples	Penicillin, Cephalosporins	Third-generation cephalosporins, Quinidine, Diclofenac	Methyldopa, Procainamide, Mefenamic acid, Fludarabine*, Cladribine*	Cephalosporins, Cisplatin, Carboplatin
Dose/duration	Large therapeutic doses/ prolonged	Very low dose on second or subsequent exposure/short	Therapeutic/ about 6 weeks	Therapeutic
Haemolysis	Extravascular Subacute	Intravascular Acute	Extravascular Mild/ subacute	Rare
DAT	IgG ± C3	C3 only	IgG only	IgG
Serum reaction	To drug-treated cells	Only in presence of drug or metabolite	To normal cells	To drug-treated cells
Eluate reaction	To drug-treated cells	Non-reactive	To normal cells	To drug-treated cells

* May change T-cell regulation.

the formation of immune complexes that are adsorbed onto the red cell membrane and activate complement. Classically, haemolysis occurs on the second or subsequent exposure to the drug and may develop within minutes or hours of drug ingestion. Severe intravascular haemolysis may occur with fever, rigors, and, in extreme cases, acute kidney injury. Several groups have reported fatal immune haemolysis with the third-generation cephalosporin, ceftriaxone, whilst others from the same group such as cefotaxime and ceftazidime have also been implicated.

Membrane modification:
Cephalosporins may cause a positive DAT by modifying red cell membrane components, resulting in a positive DAT, without causing haemolysis. Cisplatin, carboplatin and ribavirin in combination with interferon-α (IFN-α) or with pegylated IFN-α can all cause haemolytic anaemia by membrane modification. Ribavirin enters cells, is phosphorylated to ribavirin triphosphate and hydrolysed back to ribavirin in nucleated cells, which leaves the cells as the serum level declines. Red cells lack hydrolysing enzymes so the triphosphate accumulates, altering the membrane and prompting extravascular haemolysis. With ribavirin monotherapy, haemolysis may be compensated by increased marrow activity, but in combination with IFN-α, bone marrow suppression by the latter agent inhibits the compensatory response. In this setting, management involves dose reduction or supportive therapy with recombinant erythropoietin (epo), which may allow continuation of therapy in 10–30% of patients.

Autoimmune mechanism:
Methyldopa was the paradigmatic drug in drug-induced AIHA. There is a delay of some 6 weeks before the DAT becomes strongly positive due to IgG on the red cell surface. Haemolysis may be absent or trivial, although this is not true with some other drugs that produce haemolysis by this route, notably mefenamic acid (Ponstan). The antibodies usually show no Rh specificity when tested against Rh_{null} cells. It is important to note that some drugs produce haemolysis by both the immune complex and autoimmune mechanisms, depending on the circumstances.

The diagnosis of drug-induced immune haemolytic anaemia should be made in three stages: (i) diagnosis of a DAT-positive haemolytic anaemia, (ii) careful drug history and (iii) serological demonstration of drug-specific antibody, which interacts with red cells.

Carcinoma-related warm AIHA

Carcinoma-related warm AIHA has been recorded with a number of malignancies, but it remains unclear whether there is a true association between the tumour and onset of haemolysis. Reports exist in patients with ovarian cysts where the cystic fluid has been found to contain the agglutinin and in patients with ovarian carcinoma.

Viral infections

Viral infections may precede warm AIHA in children, but rarely in adults; the haemolysis is usually brisk, but self-limiting. Hypotheses include alteration of the red cell membrane by the virus, thereby generating 'auto' antibodies against altered antigens or formation of antiviral antibodies that cross-react with membrane antigens. A third possibility involves the formation of immune complexes between the virus and specific antibodies, which are secondarily adsorbed onto the red cell surface, leading to immune destruction.

Clinical management

The indication for treatment of AIHA is symptomatic anaemia, however, treatment in AIHA is largely not evidence-based.

Supportive therapy

Blood transfusion must be given if the clinical situation demands it, if the haemoglobin continues to fall or heart failure develops, despite the impossibility of achieving a satisfactory crossmatch in the presence of a positive DAT. The least incompatible grouped blood should be used and transfused slowly. Some authors recommend the use of blood-lacking antigens to which the autoantibodies react, but others point out that specificity is rarely absolute and that there is a risk of provoking an alloantibody response.

Intravenous immunoglobulin (IVIg) has been used in AIHA, particularly when IgG is the main component on the red cell surface. This is given at the same dose as used for ITP, typically 0.4 mg/kg daily for 5 days or 1 g/kg daily for 2 days. However AIHA responds less frequently to IVIg. Side effects of IVIg include headache, fever–chill reactions and a small but present risk of viral transmission. Folic acid supplements should be given to all patients with chronic haemolysis.

First-line treatment

Corticosteroids are effective in warm AIHA: 70–80% of patients will respond to prednisolone given at a dose of 1 mg/kg body weight once daily for 10–14 days. If parenteral administration is preferred, an equivalent dose of methylprednisolone can be used. In patients who respond, the dose should be reduced steadily using laboratory tests, including Hb and LDH as a guide, it is important not to stop steroids too quickly as this may result in relapse. Although 70–80% of patients improve their Hb initially on corticosteroid treatment only 30% maintain this response long term. Response rates may be improved when steroids are used in combination with rituximab, a chimeric murine–human monoclonal antibody that binds to CD20 antigen on B cells and immature plasma cells. The only two prospective randomised trials of treatment for warm AIHA both

compared prednisolone to prednisolone plus rituximab first line both showed improved responses in the combination arm at 75% versus 31 and 36% at 1 year. However neither study was powered nor had long enough follow-up to address whether the combination reduced the need for other treatments and improved long-term remissions. Rituximab remains unlicensed in AIHA treatment.

Corticosteroids, in general, are well-tolerated by patients, especially over the short term. However, when given for a prolonged period a number of side effects including weight gain, hypertension, diabetes, cardiovascular disease, osteoporosis and immunosuppression may be present. The use of pneumocystis prophylaxis is sometimes considered in patients on long-term steroid therapy.

Second-line treatments

Rituximab remains the most commonly used second-line treatment (when not used up-front). The overall response rate for rituximab from meta-analysis is around 79%, with a median time to response around 3–6 weeks, but 30% of patients who receive it first line relapse after 3 years. Re-treatment of relapsed patients gives similar results. Half the patients in reported studies received concomitant corticosteroids. Rituximab is generally well tolerated, immediate side effects are minimal and mainly infusion-related. It is associated with significant B-cell depletion which causes hypogammaglobulinaemia and prolonged immunosuppression. This can lead to infections such as reactivation of hepatitis B, hence pre-administration screening for hepatitis B is required. A further rare but significant infection risk is with JC virus which may lead to development of multifocal leukoencephalopathy – a rare and usually fatal condition.

Treatment with cytotoxic immunosuppressive drugs is reserved for steroid-refractory patients, and relapse following steroid withdrawal. The mechanism of action of these agents is relatively non-selective, resulting in significant systemic toxicity, including bone marrow suppression, renal toxicity and the potential long-term risk of malignancy. It should be emphasised that there are no controlled trials to prove the worth of cytotoxic drugs in AIHA, but there are small case reports of success with each agent. Response to these drugs is not usually seen for 4–6 weeks and they should be continued for at least 3 months before being deemed ineffective.

Azathioprine has response rates of around 55–60% in warm AIHA and it has significant steroid-sparing action at a dose of 2–2.5 mg/kg daily. Danazol has been used, both in combination with corticosteroids as a first-line treatment and in the refractory setting, with mixed results in AIHA. Other agents include cyclophosphamide 1.5–2.0 mg/kg daily and ciclosporin, starting at 2.5 mg/kg twice daily to achieve trough plasma levels of 100–200 mg/L. There are small case reports of the use of mycophenolate mofetil, an immunosuppressive agent, and of vincristine, a cytotoxic agent with powerful antitumor effect, with some success in severe refractory AIHA. Small case series have also reported some effectiveness with Bortezomib, a proteasome inhibitor used primarily in myeloma therapy.

Splenectomy

Patients with predominantly IgG on the red cell surface respond best to splenectomy, while those with complement respond poorly. Of a selected group of patients post splenectomy, about 40% achieve a complete remission and do not require steroids, and a third will show no or only transient response before relapse; the rest will have reduced steroid requirement. There is no certain way to determine clinical response to splenectomy.

Splenectomy is associated with an increased risk of overwhelming sepsis, therefore all patients should receive pneumococcal, meningococcal and *Haemophilus influenzae B* vaccination at least 2 weeks before the procedure, and prophylactic penicillin indefinitely.

Prognosis

The prognosis of warm AIHA depends on a number of variables, including age, associated diseases and severity of haemolysis. AIHA can be a serious and potentially life-threatening disease with reported estimates of mortality of idiopathic AIHA in adults of 10% at 5 years to 40% at 7 years. Increased mortality typically occurs in patients over 50 years with deaths in the first 2 years after diagnosis usually related to underlying clinical disorders or treatment side-effects. Most children with AIHA recover completely, and mortality is much lower, probably about 5%. Treatment-related side-effects, particularly prolonged high-dose steroid therapy, can result in considerable morbidity.

Cold agglutinin disease

Cold agglutinin disease (CAD) is a relatively uncommon disorder accounting for only 15–30% of AIHA; it occurs mainly in the elderly and typically runs a chronic course. CAD is mostly benign, but the clinical features can be disabling and distressing.

Clinical features of cold agglutinin disease

The clinical features of the cold agglutinin haemolytic anaemia vary with the pathogenesis of the disorder. Symptoms resulting from red cell agglutination are present in most patients and can be problematic, including purplish skin discoloration which is maximal over the extremities (acrocyanosis), most notably present in cold weather. Acrocyanosis occurs due to stasis in the peripheral circulation secondary to red cell agglutination. On warming the skin, the colour returns to normal or there is transient erythema.

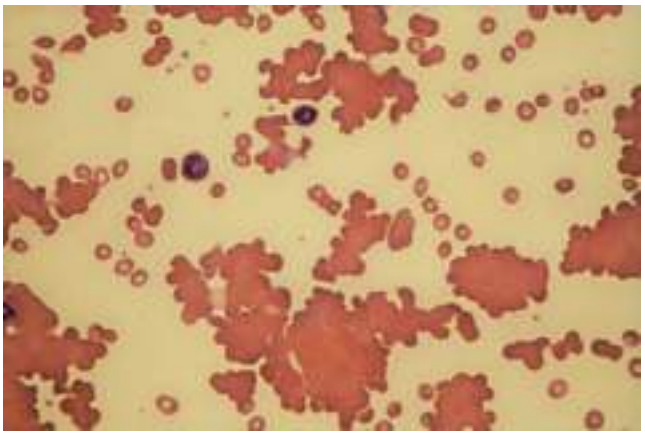

Figure 17.2 Cold haemagglutinin disease. Blood film showing gross haemagglutination (×20).

In the laboratory, spontaneous agglutination of red cells is frequently observed, both macroscopically and on the peripheral blood film if this is made at room temperature (Figure 17.2). Automated blood cell counters detect agglutinates and record erroneously high mean corpuscular volume and low Hb values, unless the sample is tested at 37 °C.

DAT is positive with only C3d on the red cell surface; IgM cold agglutinins are not detected because they elute from the cell surface *in vitro*. The serological characteristics of the antibodies found in these syndromes are shown in Table 17.3. Cold agglutinins are monoclonal IgM κ, with heavy chain variable regions encoded by the *IGHV4*–34 gene segment, but serum electrophoresis may not reveal a monoclonal band because the concentration of the protein is too low.

Traditionally defined by the absence of an underlying disorder, recent studies using sensitive tests including flow cytometry and immunohistochemical assessments have demonstrated a monoclonal CD20-positive κ-positive B-lymphocyte population in the bone marrow of 90% of patients with CAD. Therefore some authorities consider CAD a premalignant B-cell disorder that becomes clinically overt due to the specificity of the antibody for red cell surface antigens. The prognosis is usually that of the underlying lymphoproliferative disease.

Cold agglutinin syndrome

These are usually secondary to either aggressive lymphoma or specific infections, most commonly *M. pneumoniae*, or infectious mononucleosis. Rare cases following *Listeria* or *Toxoplasma* infections have also been reported. These antibodies are mostly polyclonal IgM in type, but occasional IgG cold antibodies are found. The antibodies develop in response to the infectious organism and cross-react with red cell antigens; haemolysis occurs 2–3 weeks after the infection and is usually mild and self-limiting. Acute and potentially fatal episodes of intravascular haemolysis have been reported in association with *M. pneumoniae* infection. Urgent blood transfusion through a blood warmer may be required.

Management of CAD

The management of cold haemagglutinin syndromes may be challenging and is summarised below.

Supportive therapy

The majority of patients with CAD can be managed supportively. All patients should avoid exposure to cold; the use of electrically heated gloves and socks should be encouraged in the winter months. Patients should be nursed in a warm environment, which may include measures such as special blankets during surgical procedures. In the context of infections, the anaemia may be worsen, but this can often be managed with short-term transfusion support

Table 17.3 Serological characteristics of cold-acting antibodies in the cold agglutinin syndromes.

Disorder		Antibody characteristics	
		Specificity	**Clonality and subtype**
Primary	Idiopathic (CHAD)	Anti-I Rarely Anti-P	Monoclonal IgM κ Monoclonal
Secondary	Lymphoproliferative disease	Anti-I Rarely Anti-I	Monoclonal IgM κ/λ (IgG) Monoclonal
	Mycoplasma pneumoniae	Anti-I	Polyclonal
	Infectious mononucleosis	Anti-I Anti-i	Polyclonal
Paroxysmal cold haemoglobinuria		Anti-P Donath–Landsteiner (biphasic)	Polyclonal IgG

CAD, cold agglutinin disease.

without committing to long-term systemic therapy. Blood transfusions should be given with due regard to the difficulty in cross-matching in the presence of cold haemagglutinins. Blood should be given through an in-line blood warmer.

Plasma exchange (therapeutic apheresis) can be used to control severe symptoms by temporarily lowering the titre of cold agglutinins, but it has no effect on the underlying disease. If patients are severely symptomatic despite supportive treatment then they may require systemic therapy (see below).

Management of secondary CAS is usually supportive. Transfusions are given when indicated with the same precautions as in primary CAD. The rest of the management strategy aims at treatment of the underlying condition where possible.

Folic acid supplements should be given to patients with chronic haemolysis.

Drug treatments

Unlike warm-type AIHA, corticosteroids are rarely of use in CAD and should only be used in exceptional circumstances when antibodies are present in low titres and have a high thermal range. Rituximab, the anti-CD20 monoclonal antibody has been shown to be effective in idiopathic CAD and in cold agglutinin syndromes (CAS) associated with LPD. Response rates of up to 50% have been reported in phase II studies, but with a short duration of response around 11 months. Longer response is seen with a combination of rituximab and fludarabine, with remission in 75% of patients and median duration of over 66 months. However, significant side-effects including grade 3–4 neutropenia make this regimen unsuitable for elderly patients. Rituximab and bendamustine in combination also resulted in improved response rate of 71% (40% complete remission and 31% partial remission), and only 20% had neutropenia suggesting that this regimen is more widely applicable to the older patient population.

Anaemia in CAD is caused by complement-mediated red cell lysis and complement inhibition in the treatment of CAD has shown promise. The classical pathway components responsible for extravascular haemolysis in CAD occur at the C3 level. Eculizumab is a humanised monoclonal antibody that inhibits cleavage of C5 into C5a and C5b preventing the formation of the terminal complement system including the formation of the membrane attack complex. Eculizumab will not inhibit the C3b-mediated extravascular haemolysis and therefore blockade of C5, but it was the first complement inhibitor to be used in the treatment of severe CAD, and a small trial demonstrated significant reductions in LDH, haemoglobin stabilisation and transfusion avoidance.

Blockade at the C3 level is more promising. Sutimlimab is a humanised monoclonal antibody that inhibits C1s to stop complement-mediated haemolysis and is in phase 3 trials. Similarly, Pegcetacoplan, a subcutaneously administered peptide molecule that binds C3 and C3b thereby reducing both extravascular and intravascular haemolysis, is also under investigation in CAD.

Paroxysmal cold haemoglobinuria (PCH)

This rare syndrome occurs in children following acute viral infections or childhood immunisations. A history of cold exposure is not always present and presentation is acute with significant intravascular haemolysis resulting in pallor, dark urine (haemoglobinuria), abdominal pain and sometimes collapse. The original cases were described by Donath, Landsteiner and Ehrlich in congenital and tertiary syphilis. The cold antibody is a biphasic polyclonal IgG antibody (Donath–Landsteiner antibody) that reacts with the P antigen on red cells below 20 °C in the peripheral circulation, with subsequent lysis by complement activation on rewarming to 37 °C in the central vessels. Treatment involves strict avoidance of cold and keeping the patient warm, preferably at an ambient temperature of 37 °C until the self-limiting pathological process subsides.

On the rare occasions where there is brisk haemolysis causing symptomatic anaemia such that blood transfusion is required, obtaining compatible P-negative or pp donor red blood cells for transfusion is difficult, as the P antigen is a high incidence antigen and most blood donors are P positive. Despite this *in vitro* serologic incompatibility, there are several case reports of successful transfusion of P-positive red cells to patients with PCH who then achieve a rise in Hb level without precipitation of further haemolysis. In emergency cases associated with symptomatic anaemia despite supportive measures, transfusion of ABO- and Rh-compatible P-positive blood should be used. It is important that the blood is transfused using a blood warmer or where this is not available, that the patient is kept warm and transfused slowly.

There are case reports of chronic or refractory disease, particularly in adults, where rituximab has been used successfully, and eculizumab has also been used with some success in refractory paediatric cases.

Alloimmune haemolytic anaemia with anti-D

Severe, acute intravascular haemolysis has been reported in some patients, 1–24 hours following treatment with a number of intravenous anti-D immunoglobulin preparations, which have been licensed for treatment of ITP in unsplenectomised RhD-positive patients. In this setting, higher doses are used than for prevention of haemolytic disease of the fetus and newborn. The mechanism of action is thought to be inhibition of platelet destruction by the spleen due to phagocytosis of coated red cells. Intravascular haemolysis may lead to oliguria and disseminated intravascular coagulation (DIC) and a number of deaths have been reported. The reaction is similar to that seen following mismatched transfusion reactions (see Chapter 19), but so

far no culpable antibodies other than anti-D have been detected in different batches of the anti-D preparations tested. The reaction does not happen at the doses used to prevent Rh sensitisation in pregnancy.

Non-immune acquired haemolytic anaemias

Haemolysis and haemolytic anaemia may be the consequence of a wide variety of acquired conditions that do not lend themselves to a precise and logical classification. Classification tends to be based on causes rather than mechanisms, although there are some common pathogenic mechanisms that lead to red cell destruction. The main groups of agents causing haemolysis are infections, vascular disorders (mechanical disorders), chemical and physical agents and disorders affecting the red cell membrane. A classification is shown in Table 17.4.

Infections causing haemolytic anaemia

A variety of infections may produce haemolysis through several different pathways. Haemolysis may be a consequence of direct invasion of the red cell by a microorganism or may arise from alterations in the microcirculation, leading to mechanical haemolysis. The intracellular organisms tend to produce the more severe haemolysis.

Malaria

Some degree of haemolysis is seen in all types of malarial infection, but the most severe abnormalities are found in *Plasmodium falciparum* infection (Figure 17.3). *Plasmodium falciparum* infection is one of the most common causes of anaemia in the world. Many factors may contribute to the anaemia, including marrow suppression, dyserythropoiesis, folate deficiency, hypersplenism and red cell sequestration, as well as haemolysis. The condition has two main components: extravascular destruction

Table 17.4 Non-immune acquired haemolytic anaemias.

Cause	Mechanisms	Examples
Infections	Intracellular organisms	*Falciparum* malaria Babesiosis *Bartonella*
	Endotoxin-induced DIC	Meningococcal sepsis Pneumococcal sepsis Gram-negative sepsis
	Haemophagocytic syndromes	Atypical mycobacterial infections HIV Viruses
	Enzyme toxins	*Clostridium perfringens* Snake, spider bites
Chemical and physical agents	Oxidative damage	Drugs Industrial/domestic substances
	Heat	Burns
	Osmotic lysis (fresh water), dehydration of red cells (salt water)	Drowning
	Enzyme inhibition	Lead poisoning Copper (Wilson disease)
Fragmentation (mechanical)	Lysis on prosthetic surfaces	Cardiac haemolysis Perivalvular leak
	Vasculitis, endothelial cell swelling, fibrin shear	Microangiopathic haemolytic anaemia March haemoglobinuria
Acquired membrane disorders	Lipid or cholesterol changes Somatic mutation	Liver disease Paroxysmal nocturnal haemoglobinuria (PNH)

DIC, disseminated intravascular coagulation.

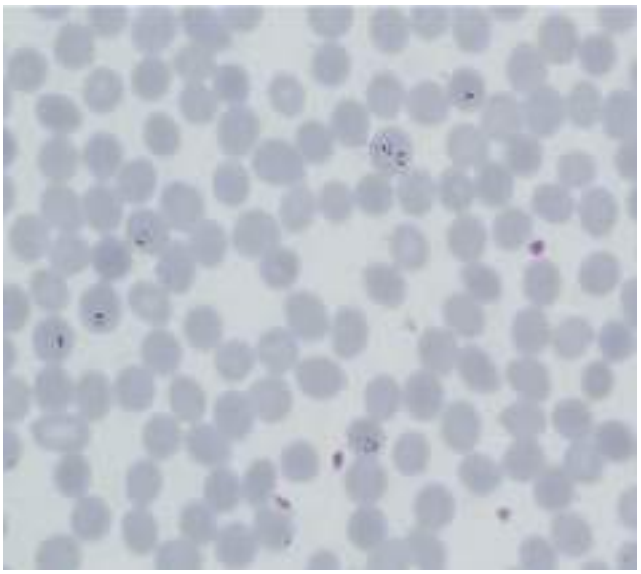

Figure 17.3 *Falciparum* malaria infection (Giemsa × 100) (Source: Dr Robin Ireland. Reproduced with permission).

of parasitised cells in the reticuloendothelial system, particularly the spleen, and intravascular lysis when the sporozoites break out of the red cells into the circulation. In most patients, the systemic symptoms of malaria dominate the clinical picture, but occasionally, acute intravascular haemolysis is the presenting problem. Haemolysis in malaria may be associated with a positive DAT.

Blackwater fever

This is an uncommon, but well-described and feared complication of *falciparum* malaria with an acute and potentially fatal episode of intravascular haemolysis leading to the passing of black or dark-red urine. Occurring after a few days of malaria fever, the patient presents with further fever and rigors, loin pain and appearance of black urine. Acute hypovolaemia and hypotension may result in oliguric acute kidney injury if not promptly managed, and in some cases, pulmonary and cerebral symptoms may develop. The condition is reported in all ethnic populations in endemic areas and does not seem to be confined to non-immune individuals. In indigenous populations, glucose-6-phosphate dehydrogenase (G6PD) deficiency may play a part in the pathogenesis in addition to quinine exposure. The spread of chloroquine-resistant malaria in the Far East led to increased use of quinine and subsequently, to an increase in the incidence of blackwater fever.

The degree of parasitaemia is very variable, and the red cell count may fall to 1×10^{12}/L within 24 hours of the start of the haemoglobinuria. There is usually a rise in fibrin degradation products in the serum, but this rise is not often marked and is compatible with a degree of kidney injury. Intravascular coagulation does not seem to play a major role in the pathogenesis.

Immediate treatment includes correction of fluid and electrolyte loss, transfusion support and eradication of the parasite. Renal dialysis may be required and it is important to note that renal supportive therapy may need to continue for a period of weeks before renal function returns. Subsequent attacks of *falciparum* malaria may produce further episodes of blackwater fever making adherence to prophylaxis mandatory in susceptible individuals.

Babesiosis

Infection with the intracellular protozoan *Babesia* is uncommon and symptomatic disease is mostly confined to splenectomised patients. *Babesia* is a tick-borne organism; the tick in Europe, *Ixodes ricinus*, is associated with cattle, whilst in North America *Ixodes dammini* is carried by rodents and deer. Transmission can also occur by blood transfusion. In splenectomised patients, the disease has an acute onset and in the European variety, can be fatal. A period of malaise for 1–3 days may be followed by vomiting, diarrhoea, high fevers, rigors, jaundice, acute intravascular haemolysis, haemoglobinuria, acute kidney injury and death. In North America, unsplenectomised patients tend to experience a milder self-limiting haemolytic anaemia. The diagnosis is made from identification of the parasites, very similar to *P. falciparum*, within the red cells in the peripheral blood film. A history of tick bite or exposure to potential vectors should be sought. Treatment is difficult. Clindamycin and quinine are standard therapy, whilst red cell exchange transfusion and renal support may be required in severely affected patients.

Bartonella (Oroya fever)

Bartonella bacilliformis is an arthropod-transmitted infection found only in the western Andes of Peru and neighbouring countries. The diagnosis is made from the peripheral blood film. The organism is an intracellular Gram-negative rod during the acute attack, which becomes coccoid in recovery. In non-immune individuals, presentation is with anaemia which occurs partly through intravascular haemolysis and partly through erythrophagocytosis as well as splenomegaly. The organism is rapidly killed by chloramphenicol, tetracyclines, penicillin and aminoglycosides.

Clostridium perfringens

Clostridium perfringens septicaemia causes an intense intravascular haemolysis with prominent microspherocytosis and ghost cells in the peripheral blood film. The spherocytosis is the result of membrane destruction by lipases and proteases produced by the organism. Although the organism is sensitive to a variety of antibiotics, the appearance of intravascular haemolysis is usually a harbinger of death because of the toxaemia.

Toxoplasmosis

Infection with *Toxoplasma gondii* acquired *in utero* may produce haemolysis and a syndrome similar to haemolytic disease of the newborn. In adults, toxoplasmosis is not associated with haemolysis, except perhaps in the immunocompromised host.

Bacterial infections

Intravascular coagulation produced by bacterial infection may be accompanied by some degree of intravascular haemolysis with fragmentation of red cells. Septicaemia from meningococcal or pneumococcal infection may result in haemolysis, which is not as clinically significant as the other effects of the septicaemia.

Haemorrhagic fevers

The haemorrhagic fevers may be accompanied by haemolysis. These include dengue fever, which is present worldwide, yellow fever, and West African haemorrhagic fevers, which are mainly found in Africa.

Fragmentation haemolysis: mechanical haemolytic anaemias

Fragmentation haemolysis may occur in the presence of prosthetic materials, due to altered flow following cardiovascular surgery, due to trapping or adherence of red cells in arteriovenous malformations and as a result of the destruction of red cells in pathologically altered small blood vessels (microangiopathic haemolytic anaemia). Characteristic features of fragmentation haemolysis are the appearance of the blood film (Figure 17.4) and the presence of intravascular haemolysis. Depending on the underlying vascular pathology, there may be a reduction in the platelet count and evidence of DIC. The rate of red cell destruction also varies according to the pathogenesis, so the signs of intravascular haemolysis vary from absence of haptoglobin, elevated LDH and minimal haemosiderinuria to acute intravascular destruction with haemoglobinaemia and haemoglobinuria. The major causes of fragmentation haemolysis are shown in Table 17.5.

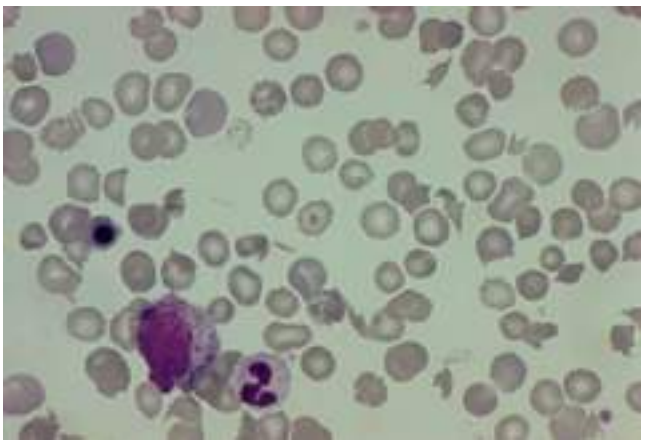

Figure 17.4 Microangiopathic haemolytic anaemia. Blood film from a patient with carcinoma and bone marrow metastases. Note fragmentation of red cells, low platelets and leucoerythroblastic changes (circulating nucleated red cell and metamyelocyte) suggestive of underlying disseminated intravascular coagulation (×40).

Table 17.5 Classification of anaemias caused by fragmentation haemolysis.

Vascular origin	Pathogenesis	Platelets
Cardiac haemolysis	Prosthetic heart valves	Normal
	Patches, grafts	
	Periprosthetic or perivalvular leaks	
Arteriovenous malformations	Kasabach–Merritt syndrome	Very low
	Malignant haemangioendotheliomas	
Microangiopathic (see also Table 17.6)	TTP/HUS	Low
	Malignant disease	Normal/low
	Vasculitis	Normal/low
	Pre-eclampsia, HELLP	Low
	Renal vascular disorders	Normal/low
	Disseminated intravascular coagulation	Low

HELLP, haemolysis with elevated liver function tests and low platelets; HUS, haemolytic–uraemic syndrome; TTP, thrombotic thrombocytopenic purpura.

Haemolysis associated with cardiac surgery

Cardiac haemolytic anaemia was a term coined to describe haemolysis following cardiac surgery that involved the insertion of prosthetic valves, patches or grafts. Mechanical trauma to red blood cells is the primary cause of haemolysis in this setting and is mainly due to increased turbulent flow resulting in excessive shearing forces on the surface of the red cells. Secondary physiologic mechanisms include pressure fluctuations, intrinsic abnormalities of the red cell membrane (largely due to fragile, iron-poor red cells in iron-deficient patients), interaction with foreign surfaces and unfavourable flow characteristics of valves. Clinical situations in which cardiac haemolysis may be of considerable importance include:

- *Periprosthetic or perivalvular haemolysis* – following insertion or repair of a heart valve, a leak occurs around the valve or through a suture track, which may cause severe intravascular haemolysis without haemodynamic distress. Laboratory diagnosis may be difficult because red cell fragmentation may not be prominent on the blood film, although spherocytes may be present.

The diagnosis is made by exclusion of an autoimmune haemolysis in a patient who experiences intravascular haemolysis following recent cardiac surgery. The haemolysis can only be cured by further surgery.

- *Ambulatory haemolysis* occurs when a patient who has undergone valve replacement shows evidence of mild haemolysis while in hospital but experiences significant anaemia after discharge. The higher cardiac output associated with greater exercise following discharge from hospital produces greater turbulence and results in red cell fragmentation. A similar mechanism may occur if the patient becomes iron deficient as a result of chronic intravascular haemolysis. Iron replacement and advice about slowly increasing exercise levels may prevent the need for further surgery.
- *Cardiopulmonary postperfusion syndrome* – acute intravascular haemolysis may occur in patients who have undergone cardiopulmonary bypass surgery. The haemolysis may be accompanied by neutropenia and pulmonary distress. Although clearly relevant to patients with cardiac pathology, this syndrome is not strictly classified as cardiac haemolysis since the haemolysis seems to be caused by complement activation and binding of the membrane attack complex to the red cell surface. The blood film shows ghost red cells rather than fragmentation. The condition is self-limiting and the patient requires only supportive care.

Arteriovenous malformation

Fragmentation of red cells may be seen in Kasabach–Merritt syndrome, in which platelets are trapped in the vascular network of giant arteriovenous malformations, sometimes with evidence of a consumption coagulopathy. The bleeding disorder that ensues is of greater significance than haemolysis in these patients. A similar pattern is seen in malignant haemangioendothelioma, in which the tumour invades and grows alongside veins resulting in a consumptive coagulopathy with laboratory evidence of DIC.

Microangiopathic haemolytic anaemias (MAHA)

These are conditions in which intravascular haemolysis with fragmentation of red cells is caused by their destruction in an abnormal microcirculation. Proof of microangiopathy may be lacking in those not subjected to a post-mortem examination, and MAHA should be considered a clinical syndrome. The three main pathological lesions that give rise to MAHA are deposition of fibrin strands, often associated with DIC, platelet adherence and aggregation, and vasculitis. The vessel abnormalities may be generalised or confined to particular sites or organs. In most cases, haemolysis is of less consequence than the underlying cause of the microangiopathy, but fragmentation of red cells helps to confirm the diagnosis. Some of the disorders producing MAHA are given in Table 17.6 and the more well-defined clinical syndromes are described in detail here.

Table 17.6 Causes of microangiopathic haemolytic anaemia.

Disease	Microangiopathy
Haemolytic–uraemic syndrome	Endothelial cell swelling, microthrombi in renal vessels
Thrombotic thrombocytopenic purpura	Platelet plugs, microaneurysms, small-vessel thrombi
Renal cortical necrosis	Necrotising arteritis
Acute glomerular nephritis	
Malignant hypertension	
Pre-eclampsia	Fibrinoid necrosis
HELLP	
Polyarteritis nodosa	Vasculitis
Granulomatosis with polyangiitis	
Systemic lupus erythematosus	
Homograft rejection	Microthrombi in transplanted organ
Mitomycin C	Uncertain
Ciclosporin	Renal vessel anomalies
Carcinomatosis	Abnormal tumour vessels, intravascular coagulation (disseminated or localised)
Primary pulmonary hypertension	Abnormal vasculature
Cavernous haemangioma (Kasabach–Merritt)	Local vascular changes, thrombosis

HELLP, haemolysis with elevated liver function tests and low platelets.

MAHA and malignant disease

Fragmentation of red cells with chronic intravascular haemolysis may occur in malignant disease. Clinically significant anaemia may occur, especially when the tumour invades large blood vessels, as occurs in haemangiopericytoma, but more commonly haemolysis is trivial or well-compensated. Other malignancies closely associated with MAHA include mucin-secreting tumours and acute leukaemia, particularly, but not exclusively, acute promyelocytic leukaemia, where acute intravascular coagulation occurs and dominates the clinical picture. A blood film showing evidence of MAHA together with leucoerythroblastic changes may be suggestive of malignant disease with secondary deposits in the bone marrow (Figure 17.4).

MAHA and infection

Infections, particularly septicaemia, may provoke intravascular coagulation and MAHA. Generally, the coagulation changes and septic shock overshadow the mild fragmentation, but occasionally infections produce a chronic state of partially compensated intravascular haemolysis and marked red cell

fragmentation. Haemolytic–uraemic syndrome (HUS) may be precipitated by infection, particularly *Escherichia coli* O157.

Thrombotic thrombocytopenic purpura

Thrombotic thrombocytopenic purpura (TTP) is an acute syndrome characterised by fever, neurological signs, haemolytic anaemia with fragmented red cells and profound thrombocytopenia (Chapter 46). There is severe deficiency of von Willebrand factor cleaving protease (also known as *ADAMTS13*) to less than 10% of normal activity due to formation of auto-antibodies directed against it. The diagnosis is made on the basis of the clinical presentation and evidence for haemolytic anaemia with fragmented red cells and thrombocytopenia.

March haemoglobinuria

Haemoglobinuria following running has been documented for about 100 years. Its origin is mechanical, with destruction of red cells occurring in the feet. It can be cured by wearing soft shoes or running on soft ground. The disorder may arise in joggers and is benign except that it may lead to extensive invasive investigations unless recognised. The blood film does not show any red cell fragmentation or consistent abnormality. Occasionally, haemoglobinuria after running is accompanied by nausea, abdominal cramps and aching legs and enthusiastic athletes with this condition may exhibit mild splenomegaly and jaundice.

Chemical and physical agents

Oxidative haemolysis

Oxidative substances may cause haemolysis in people with normal red cell metabolism and normal HbA if the oxidative stimulus is large enough. The major causes of oxidative haemolysis in normal subjects are shown in Table 17.7. The clinical features of this condition are dependent on the main sites of oxidative attack: whether on the membrane of the red cell (intravascular haemolysis), the globin chains (Heinz body formation) or the haem group (methaemoglobin accumulation).

Dapsone and sulfasalazine (Salazopyrin) will cause oxidative intravascular haemolysis in normal subjects if taken in sufficiently high dosage. Normal doses will cause significant oxidative haemolysis when given to G6PD-deficient patients. The blood film shows polychromasia, macrocytosis and the classical 'bite' abnormality of the chemically damaged cell (Figure 17.5). Heinz bodies appear on the blood film when G6PD haemolysis occurs in hyposplenic individuals, but they are absent or scanty in patients with an intact spleen. Haemolysis is usually well-compensated and there is no need to stop the treatment unless the anaemia is severe. Methaemoglobinaemia is uncommon unless the patient is partially deficient in NADH methaemoglobin reductase.

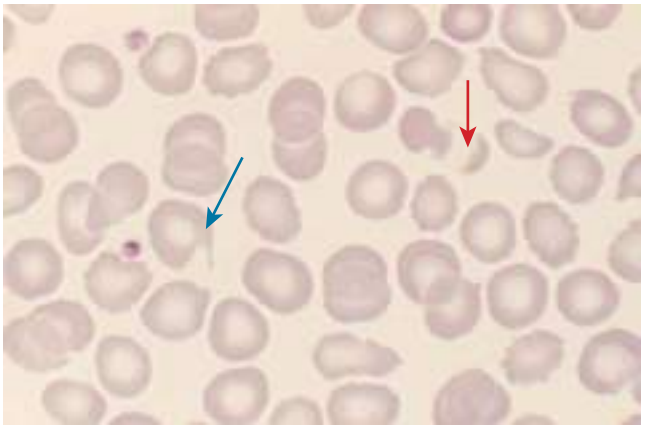

Figure 17.5 Oxidative haemolysis caused by drug (phenacetin). Note red cells with bite cells (blue arrow) and contracted haemoglobin (red arrow).

Table 17.7 Substances causing oxidative haemolysis and/or methaemoglobinaemia in people with normal red cell metabolism.

Substance	Use	Remarks
Dapsone	Leprosy, dermatitis herpetiformis	Chronic haemolysis; slow acetylators more susceptible
Maloprim	Antimalarial	Methaemoglobinaemia in NADH methaemoglobin reductase-depleted subjects
Sulfasalazine	Ulcerative colitis	Chronic intravascular haemolysis
Phenazopyridine	Analgesic in urinary tract infections	Methaemoglobinaemia
Menadiol	Water-soluble vitamin K analogue	Haemolysis/kernicterus in infants
Nitrites	Fertiliser; present in well water, some vegetable juices	Methaemoglobinaemia in infants
Nitrates	Amyl nitrate, butyryl nitrite; abused recreationally	Methaemoglobinaemia/haemolysis
Chlorate	Weed-killer	Acute intravascular haemolysis; acute kidney injury; >30 g fatal
Arsine	Gas produced in smelting and other industrial processes	Acute intravascular haemolysis; renal failure

Methaemoglobinaemia

Methaemoglobinaemia may be caused by nitrites in water and by vegetable juices in infants with physiological impairment of the reducing systems. Well water that comes from land with an excess of nitrites and which is used to reconstitute artificial feeds has produced cyanosis in infants. Cases have also been described following the enthusiastic feeding of juice from carrots grown on organically fertilised land and of spinach juice (spinach has a high concentration of nitrogen-fixing bacteria on its leaves). Nitrate drugs, for example, amyl nitrate, also produce methaemoglobinaemia and have proved fatal when taken in sufficiently high dosage for 'recreational' purposes. Water-soluble analogues of vitamin K (menadiol sodium diphosphate) cause haemolysis with or without methaemoglobinaemia in infants and *in utero* if given to the mother during the third trimester. Fat-soluble vitamin K preparations must be used if required in these situations. Methaemoglobinaemia due to oxidative drugs rarely causes problems with oxygen delivery, but severe cases (>40% methaemoglobin) may be treated with intravenous methylene blue 1–2 mg/kg. Ascorbic acid by mouth may also be used. These measures are ineffective in G6PD-deficient patients and when very strong oxidant substances are implicated. In these circumstances, methylene blue should be avoided because it acts as an oxidant and makes the condition worse.

Methaemoglobinaemia following exposure to strong oxidising substances may cause acute intravascular haemolysis and acute kidney injury. These substances are found mainly in industrial or horticultural settings and include sodium chlorate, a weed-killer, and arsine, a gas produced in various industrial situations such as dross removal in smelting procedures and drain clearance where arsenic is a contaminant. Acute intravascular haemolysis and haemoglobinuria develop 1–24 hours after exposure, depending on the dose. The serum becomes brown, often very dark, so that blood cells cannot be seen in anticoagulated preparations, due to the presence of methaemalbumin, methaemoglobin and free haemoglobin. Vomiting, abdominal pain and oliguric acute kidney injury usually develop over about 24 hours. The blood film shows microspherocytosis, ghost cells and bizarre forms. Urgent treatment is required with red cell exchange and renal dialysis, methylene blue is ineffective.

Thermal injury

Normal red cells when heated *in vitro* to 46 °C for 1 hour show no changes, however they show temperature- and duration-dependent changes above 47–50 °C. Some hereditary membrane defects produce red cells that have increased thermal fragility.

Severe burns

Severe burns may be accompanied by intravascular haemolysis with haemoglobinuria. The intravascular haemolysis is related to the extent and severity of the burns. The gross haemoglobinuria occurs over the first 24 hours after the burns and ceases thereafter. The blood film shows spherocytosis and schistocytes, the morphological abnormalities reflecting the thermal damage and the amount of lysis. Prolonged anaemia after burning is related to inflammation, occult blood loss and infection rather than haemolysis.

Lead poisoning

Haemolysis is an important contributor to the anaemia associated with excessive lead exposure. Lead is a potent inhibitor of pyrimidine 5′-nucleotidase 1 (P5N1). This is the prime mechanism of lead-induced haemolysis, producing the same gross basophilic stippling of the red cells as seen in the inherited deficiency of the enzyme (see Chapter 16). P5N1 activity is a good surrogate marker for lead exposure.

Acquired disorders of the red cell membrane

The most common acquired disorder is paroxysmal nocturnal haemoglobinuria (PNH), caused by somatic mutation of the phosphatidylinositol glycan A (*PIGA*) gene on the X chromosome, which leads to failure to produce the glycosylphosphatidylinositol (GPI) anchor needed to transport and attach many proteins to the red cell membrane. Intravascular haemolysis occurs through the unchecked action of activated complement.

Some degree of shortening of red cell survival occurs in most cases of acute hepatitis, cirrhosis and Gilbert disease, but anaemia is not present and there is only a slight rise in reticulocytes. Biliary obstruction is associated with the appearance of target cells and fulminant hepatitis with acanthocytosis, both due to changes in plasma lipid composition.

The mature red cell does not have the capacity to repair its membrane, the lipids of the membrane are in equilibrium with the lipids of the plasma and changes in the ratio of free cholesterol to phospholipids in plasma may affect red cell shape and, in some instances, lead to haemolysis. Although most commonly seen in liver disease, other inherited lipid disorders may also affect the red cell secondarily.

Zieve syndrome

Zieve syndrome is an uncommon disorder seen mainly in individuals with alcohol use disorder. It comprises intravascular haemolysis and acute abdominal pain in patients with jaundice and cirrhosis. The cause is unknown but is probably related to lipid changes in the blood. Spherocytes are seen in the peripheral blood.

Wilson disease

Wilson disease may present as acute intravascular haemolysis. This is probably not a membrane disorder but is consequent on the high levels of copper ions in the blood. The haemolysis may antedate development of hepatic or neurological features, but Kayser–Fleischer rings are usually present. The blood film may show spherocytosis. The diagnosis is made once the condition is suspected. Apart from caeruloplasmin deficiency, patients have a specific aminoaciduria.

Hereditary acanthocytosis (abetalipoproteinaemia)

This rare inherited deficiency of low-density lipoproteins is characterised by retinitis pigmentosa, steatorrhoea, ataxia and intellectual disability. The haemolysis that occurs is of minor importance to such patients, but the blood film may indicate the diagnosis, with the red cells showing marked acanthocytosis.

Vitamin E deficiency

Deficiency of vitamin E may occur in premature and other infants who are fed a commercial formula that is rich in polyunsaturated fatty acids. Vitamin E is an antioxidant and oxidative damage to the red cell membrane is thought to be the cause of the haemolysis. Clinically, generalised oedema associated with haemolytic anaemia is reported in association with low levels of vitamin E in premature infants aged between 4 and 8 weeks. There is laboratory evidence of haemolysis with contracted red cells and a thrombocytosis, all of which are corrected following adequate vitamin E replacement therapy.

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CHAPTER 18

Red cell immunohaematology

18

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Introduction

Red cell immunohaematology is the study of the interactions between blood group antigens and their corresponding antibodies, both *in vivo* and *in vitro*, and of the presence or absence of clinical consequences following those interactions. It encompasses: (i) determination of the phenotype of red cells from donors and patients, defined by antibodies, (ii) the search for and identification of antibodies in patients' sera, with red cells of known phenotype, (iii) in the presence of clinically significant antibodies, compatibility testing of patients' sera against selected cell samples from ABO compatible donor units and (iv) investigation of adverse effects due to incompatibility.

The aim of this chapter is to provide an introduction to blood group serology, to include aspects of immunology, biochemistry and molecular genetics that contribute to our understanding of blood group antigens, antibodies and antigen–antibody reactions, and to provide some details on the blood group systems of most relevance to blood transfusion.

Blood group systems

The International Society of Blood Transfusion recognises 393 red cell surface antigens, 366 of which belong to one of 47 blood group systems (Table 18.1). Each system represents either a single gene or two or three very closely linked homologous genes. Each system is genetically discrete from all others. In addition, there are 27 antigens that have not been included in systems, owing to inadequate genetic evidence. Most blood groups are inherited as Mendelian characteristics, although environmental factors may occasionally affect blood group expression. The 47 systems represent a total of 52 genes: MNS contains 3 loci, Rh, Xg and Ch/Rg 2 loci each, and the other 43 systems each contain a single gene. The majority of the genes are autosomal, except for *XG*, *XK*, and *ATP11C* which are on the X chromosome, and *CD99*, which is located on both the X and Y chromosomes. All 52 genes have been identified and sequenced.

The red cell membrane and chemistry of blood group antigens

The red cell membrane is composed of about 40% (w/w) lipids and up to 10% carbohydrates, the remainder being protein. Blood group antigens may be integral proteins or glycoproteins of the red cell membrane, or they may be membrane glycolipids. In proteins and glycoproteins, blood group polymorphisms and variants may represent differences in the amino acid sequence (e.g. Rh and Kell blood group systems). In glycoproteins and glycolipids, the blood group activity may reside in the carbohydrate moiety and polymorphism is associated with differences in the oligosaccharide sequence (e.g. ABO). In some glycoproteins, blood group polymorphism may be caused by amino acid substitutions, but antigen expression is also dependent on glycosylation of the polypeptide.

The integral proteins of the red cell membrane penetrate the lipid bilayer and, in some cases, are bound to the membrane skeleton or cytoskeleton. Cell-surface proteins are not necessarily discrete units within the cell membrane; they may congregate

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 18.1 Human blood group systems.

No.	Name	Symbol*	No. of antigens	HGNC gene symbol (s)	Chromosome	Structure	CD number
001	ABO	ABO	4	*ABO*	9	Carbohydrate	
002	MNS	MNS	50	*GYPA*, *GYPB*, *GYPE*	4	Glycoprotein	CD235a/b
003	P1PK	P1PK	3	*A4GALT*	22	Carbohydrate	CD77
004	Rh	RH	56	*RHD*, *RHCE*	1	Protein	CD240D/CE
005	Lutheran	LU	28	*BCAM*	19	Glycoprotein	CD239
006	Kell	KEL	38	*KEL*	7	Glycoprotein	CD238
007	Lewis	LE	6	*FUT3*	19	Carbohydrate	
008	Duffy	FY	5	*ACKR1*	1	Glycoprotein	CD234
009	Kidd	JK	3	*SLC14A1*	18	Glycoprotein	
010	Diego	DI	23	*SLC4A1*	17	Glycoprotein	CD233
011	Yt	YT	6	*ACHE*	7	Glycoprotein	
012	Xg	XG	2	*XG*, *CD99*	X/Y	Glycoprotein	CD99†
013	Scianna	SC	9	*ERMAP*	1	Glycoprotein	
014	Dombrock	DO	10	*ART4*	12	Glycoprotein	CD297
015	Colton	CO	4	*AQP1*	7	Glycoprotein	
016	Landsteiner–Wiener	LW	4	*ICAM4*	19	Glycoprotein	CD242
017	Chido–Rodgers	CH/RG	10	*C4A*, *C4B*	6	Glycoprotein	
018	H	H	1	*FUT1*	19	Carbohydrate	CD173
019	Kx	XK	1	*XK*	X	Protein	
020	Gerbich	GE	13	*GYPC*	2	Glycoprotein	CD236
021	Cromer	CROM	20	*CD55*	1	Glycoprotein	CD55
022	Knops	KN	13	*CR1*	1	Glycoprotein	CD35
023	Indian	IN	6	*CD44*	11	Glycoprotein	CD44
024	Ok	OK	3	*BSG*	19	Glycoprotein	CD147
025	Raph	RAPH	1	*CD151*	11	Glycoprotein	CD151
026	John Milton Hagen	JMH	8	*SEMA7A*	15	Glycoprotein	CD108
027	I	I	1	*GCNT2*	6	Carbohydrate	
028	Globoside	GLOB	3	*B3GALNT1*	3	Carbohydrate	
029	Gill	GIL	1	*AQP3*	9	Glycoprotein	
030	RHAG	RHAG	6	*RHAG*	6	Glycoprotein	CD241
031	Forssman	FORS	1	*GBGT1*	9	Carbohydrate	
032	JR	JR	1	*ABCG2*	4	Glycoprotein	CD338
033	Lan	LAN	1	*ABCB6*	2	Glycoprotein	
034	Vel	VEL	1	*SMIM1*	1	Glycoprotein	
035	CD59	CD59	1	*CD59*	11	Glycoprotein	CD59
036	Augustine	AUG	4	*SLC29A1*	6	Glycoprotein	
037	KANNO	KANNO	1	*PRNP*	20	Glycoprotein	CD230
038	Sid	SID	1	*B4GALNT2*	17	Carbohydrate	
039	CTL2	CTL2	5	*SLC44A2*	19	Glycoprotein	
040	PEL	PEL	1	*ABCC4*	13	Glycoprotein	
041	MAM	MAM	1	*EMP3*	19	Glycoprotein	
042	EMM	EMM	1	*PIGG*	4	Glycoprotein	
043	ABCC1	ABCC1	1	*ABCC1*	1	Glycoprotein	
044	Er	ER	5	*PIEZO1*	16	Glycoprotein	
045	CD36	CD36	1	*CD36*	7	Glycoprotein	CD36
046	ATP11C	ATP11C	1	*ATP11C*	X	Phosphoprotein	
047	MAL	MAL	1	*MAL*	2	Lipoprotein	

HGNC, Human Genome Organisation Gene Nomenclature Committee.
* ISBT gene name in italics.
† *CD99* product.

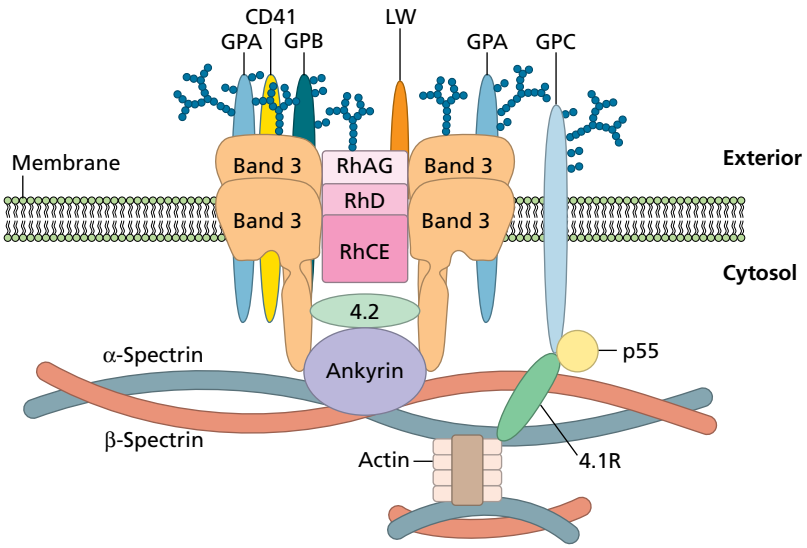

Figure 18.1 Diagrammatic representation of some integral red cell membrane proteins, including the band 3/Rh macrocomplex and components of the membrane cytoskeleton.

together in lipid rafts or might be part of complexes of proteins in which the components contribute to the function of the whole (Figure 18.1). There are four types of integral proteins in the red cell membrane. Type 1 glycoproteins, typified by the glycophorins, carrying the MNS antigens, cross the membrane once, with the amino-terminal domain on the outside and the carboxy-terminal domain in the cytosol. The Kell glycoprotein is a type 2 structure. It also crosses the membrane once but has the carboxy-terminal domain on the outside and the amino-terminal domain in the cytosol. Type 3 glycoproteins are polytopic: they cross the membrane several times giving rise to a series of extracellular loops, one of which is usually glycosylated. These N-glycans express ABO, H and Ii blood group activity. In most of these type 3 proteins, both terminal domains are cytosolic (e.g. Rh proteins, band 3, glucose transporter, Kidd glycoprotein), but the Duffy glycoprotein has an extracellular amino-terminal domain. Type 5 membrane glycoproteins, which include the Dombrock and Cromer glycoproteins, reside in membrane microdomains rich in cholesterol known as lipid rafts and are attached to the lipid bilayer by a glycolipid, the glycosylphosphatidylinositol (GPI) anchor. There are no Type 4 proteins in the red cell membrane.

On the inside of the lipid bilayer is a network of glycoproteins that comprise the cytoskeleton, which is responsible for maintenance of the shape and integrity of the red cell as it squeezes through the tiniest capillaries. The main components of the membrane skeleton are the α- and β-subunits of spectrin, which form long flexible rods, plus actin, ankyrin, proteins 4.1R and 4.2, and several other glycoproteins. Band 3, the anion exchanger and Diego blood group protein, has an extended N-terminal domain attached to the membrane skeleton through ankyrin and proteins 4.2 and 4.1R. Glycophorin (GP) C and GPD, the Gerbich glycoproteins, have C-terminal domains attached to the membrane skeleton through protein 4.1R and p55. In addition, there is evidence that several other integral membrane proteins interact with components of the membrane skeleton. Absence or reduced levels of these integral membrane proteins that interact with the membrane skeleton often result in some degree of abnormal red cell morphology (see Chapter 16).

ABH and Ii antigens are found predominantly on the carbohydrate moieties of the major red cell glycoproteins band 3 (anion exchanger) and on the glucose transporter, although they are also present on type 3 and some other minor glycoproteins and on the carbohydrate portions of membrane glycolipids. P, P1 and P^k antigens are expressed on the carbohydrate of glycolipids. The M and N antigens arise from interactions between the carbohydrate and polypeptide in glycophorin A. In addition to the antigens on integral membrane components, the Lewis and Chido/Rogers antigens are not of erythroid origin, but are adsorbed from the plasma.

Blood group antibodies

Several terms have been used in the past and are still sometimes used to describe different types of blood group antibodies, including 'naturally occurring' and 'immune' antibodies, and 'cold' and 'warm' antibodies. They are described below and an attempt is made to correlate these terms with the class of immunoglobulin involved.

Naturally occurring and immune antibodies

Antibodies are naturally occurring when they are produced without any obvious immunising stimulus, such as pregnancy or transfusion. These antibodies are not present at birth and, in the case of anti-A,B anti-A and anti-B, start to appear in the serum at about 3–6 months of age. ABO antibodies are probably

produced in response to antigens of bacteria, viruses and other substances that are inhaled or ingested. Despite this probable antigenic stimulus, the term 'naturally occurring' is retained for these 'non-red-cell-induced' antibodies. 'Immune' red cell or alloantibodies are only produced after pregnancy or following transfusion or injection of blood or blood group substances.

Cold and warm antibodies

Cold antibodies give higher agglutination titres at low temperatures (0–4 °C), and many of them will not agglutinate red cells at 37 °C. Most naturally occurring antibodies are cold-reacting. Some, such as naturally occurring anti-A,B have a wide thermal range and will react at 37 °C, activating complement and leading to red cell lysis. However, the titre will be much higher at 0–4 °C. Cold antibodies that fail to react above 30 °C are of no clinical significance and can be ignored for blood transfusion purposes.

Immune antibodies have a thermal optimum of 37 °C. Any red cell antibody reacting above 30 °C should be considered potentially capable of destroying red cells *in vivo*.

IgM and IgG

IgM antibodies agglutinate red cells suspended in saline. They are often called saline or directly agglutinating antibodies. Conversely, IgG antibodies do not usually agglutinate saline-suspended red cells. However, lack of agglutination does not mean that the antibodies have not bound to their antigen, and it can be shown that they have reacted by using antiglobulin reagents, which facilitate agglutination of antibody-coated cells (see below). Most naturally occurring antibodies are IgM and preferentially cold-reacting, whereas most immune antibodies are IgG and warm-reacting, although some may be IgM. Exceptionally, very potent IgG antibodies may be directly agglutinating.

Monoclonal antibodies

Monoclonal antibodies have increased the repertoire of well-standardised blood grouping reagents of guaranteed quantity, quality and potency. By fusing the spleen cells of immunised mice or rats with drug-sensitive myeloma cells and selecting for drug-resistant hybrids, it has been possible to establish permanently growing cloned cell lines in tissue culture that secrete antibodies of desired specificities. Several murine hybrids secreting potent human blood-group-specific monoclonal antibodies have now been established, many of which were raised using immunogens other than intact red cells (e.g. anti-A, -B, -Le^a, -Le^b, -M and -N).

Human monoclonal antibodies specific for D, other Rh antigens, Jk^a, Jk^b, and several other antigens, have been obtained by transformation of isolated peripheral blood lymphocytes from immunised individuals with Epstein–Barr virus or by fusion of human lymphocytes with mouse myelomas to form heteromyelomas.

Monoclonal antibodies of murine and human origin have widely replaced polyclonal blood grouping reagents in everyday ABO and D grouping. The future of blood grouping may lie in DNA-based array technology, which is able to provide a means of accurate extended typing of both patients and donors, where suitable serological reagents are currently unavailable.

Lectins

Lectins are sugar-binding proteins, mostly extracted from plants and lower vertebrate animals. They are useful tools for routine and experimental blood group serology. They combine with simple sugars (e.g. fucose, galactose and *N*-acetylgalactosamine) present on the glycolipids and glycoproteins of cell membranes and body fluids. The three most commonly used blood-group-specific lectins are extracts from *Dolichos biflorus*, *Vicia graminea* and *Ulex europaeus*, which have anti-A1, -N and -H specificities, respectively. Several other lectins have proven valuable in investigating red cell polyagglutinability. Lectins are also used for determining ABH secretor status and for partially purifying and identifying blood-group-active membrane glycoproteins.

Clinical significance of red cell antibodies

The clinical significance of red cell antibodies depends partly on their destructive capacity *in vivo* and partly on their frequency. ABO and D antibodies are by far the most significant. Several factors influence immune red cell destruction *in vivo*:

1 *Plasma concentration and avidity of the antibody*.

2 *Thermal amplitude of the antibody*. Only antibodies reacting at 37 °C can cause haemolysis *in vivo* because complement can only be fixed at such a temperature and because antibody binding to Fc receptors can only take place at 37 °C.

3 *Immunoglobulin class and subclass*. The complement-fixing ability of most warm-reacting IgM antibodies, such as anti-A,B, anti-A and anti-B, makes them clinically significant. Of the IgG subclasses, IgG1 and IgG3 have clinical importance because of their capacity to bind to the Fc receptors of mononuclear phagocytic cells, the effector cells of extravascular immune red cell destruction. In addition, some IgG1 and IgG3 antibodies can fix complement up to C3b.

4 *Antibody specificity*. Several warm-reacting antibodies are incapable of causing *in vivo* red cell destruction (e.g. anti-Ch, -Rg, -Cs^a, -Kn^a, -Xg^a and most examples of anti-Yt^a).

5 *Antigen density on the red cell membrane*. The likelihood and degree of sensitisation of a red cell with antibody and/or complement increases with the number of antigen sites on the surface. There are many more A sites than D sites on red cells.

6 *Volume of incompatible red cells transfused*. A small volume of incompatible red cells will be destroyed more rapidly than a large volume from the same donor. If antibodies are not very potent, larger volumes of cells may exhaust the circulating antibody available and saturate the mononuclear phagocytic system.
7 *Presence of antigen in donor plasma*. Lewis antigens (Le^a and Le^b) and the Ch/Rg antigens are primarily in plasma and are only secondarily adsorbed onto red cells. ABH antigens are present on red cells and plasma. The free antigen in plasma of transfused red cells can react with part of the recipient's antibody and inhibit its binding to red cells.
8 *Activity of cells of the mononuclear phagocyte system*. The ability of macrophages to remove sensitised red cells varies between individuals. Splenectomy and drugs such as corticosteroids and immunosuppressants will decrease the clearance of IgG-sensitised cells.
9 *Sensitivity of red cells to complement*.
10 *Extent of complement activation*. Some antibodies regularly bind complement and others do so rarely or not at all. Of the complement-binding antibodies, IgM (e.g. anti-A,B, -A, -B, -Le^a and -$PP1P^k$) will activate the complement cascade through to C9, resulting in intravascular haemolysis, but with IgG antibodies, such as anti-Fy^a, -Jk^a and -K, the cascade is interrupted at the C3 stage. Red cells coated with IgG and C3b will be destroyed extravascularly in the liver. As a rule, Rh antibodies do not fix complement.

Detection of red cell antigen–antibody reactions

Agglutination techniques

There are various ways of detecting antigen–antibody reactions *in vitro*, either manually or with automated methods. In manual methods, tubes, microplates or gels can be used. The most widely used methods employ the following techniques.

Direct agglutination

Most IgM antibodies will directly agglutinate saline-suspended red cells of the appropriate phenotype. This method is used routinely for ABO grouping with polyclonal or monoclonal antibodies and RhD typing with monoclonal antibodies.

Indirect agglutination

Apart from ABO, antibodies against most blood group antigens are IgG and generally will not produce direct agglutination of red cells. Such antibodies can be detected by the antiglobulin technique or with the aid of agents that enhance agglutination, for example proteases, such as papain, albumin and other colloids, aggregating agents such as polybrene and polyethylene glycol (PEG), and low ionic strength solutions (LISS).

Antiglobulin test

The antiglobulin test is used to detect IgG antibodies that do not cause direct agglutination of saline-suspended red cells. The technique can be used to test directly, with an antiglobulin reagent, for the presence of antibodies or complement components that are bound to the red cells *in vivo*, as in autoimmune haemolytic anaemia, haemolytic transfusion reactions (HTRs) or haemolytic disease of the fetus and newborn (HDFN); this is the so-called direct antiglobulin test (DAT). Alternatively, the test can be used before transfusion to detect IgG antibodies in a patient's serum by adding the appropriate screening test red cells and then, after incubation and thorough washing (except in microcolumn tests), adding an antiglobulin reagent that will agglutinate cells coated *in vitro* with antibody or complement components; this is the indirect antiglobulin test (IAT). If IgG antibodies are found, these will be identified by IAT with an extended red cell panel. The IAT will then be used with the appropriate antigen-negative cells in the crossmatch, to ensure compatibility between the patient's serum and donors' red cells. IATs are also used with many reagent antibodies, such as anti-K, -Fy^a, and -Jk^a for determining blood group phenotypes (Figure 18.2).

The first step in the antiglobulin test is the antibody uptake during the incubation of antibody with red cells carrying the antigen (Figure 18.2). When an IAT is undertaken with red cells suspended in normal-ionic-strength saline (NISS) solution, maximum antibody uptake, and hence maximum sensitivity, is achieved within 60–90 minutes incubation. The incubation phase can be reduced to 10–15 minutes by using low-ionic strength saline (LISS) solutions. When the DAT and IAT are performed in tubes or microplates, it is essential that, after the antibody uptake, the red cells are washed 3 or 4 times with a large volume of saline before adding the antiglobulin reagent, as any free IgG or complement will neutralise the anti-IgG or anticomplement reagent and lead to false-negative reactions. With microcolumns containing a matrix of either a gel or glass beads, the wash phase has been eliminated.

Antibodies against IgG and complement should always be present in polyspecific antiglobulin reagents. The anti-IgG component is essential for pretransfusion antibody screening and cross-matching, as the vast majority of clinically significant antibodies, apart from anti-A,B, -A and -B, are IgG. The anti-complement component is needed for the detection of occasional examples of weak complement-binding antibodies (e.g. some anti-Jk^a) and for the detection of *in vivo* complement coating of red cells (i.e. in the DAT). Antiglobulin reagents are generally produced from polyclonal antibodies to IgG, made in experimental animals, and with monoclonal antibodies to C3d.

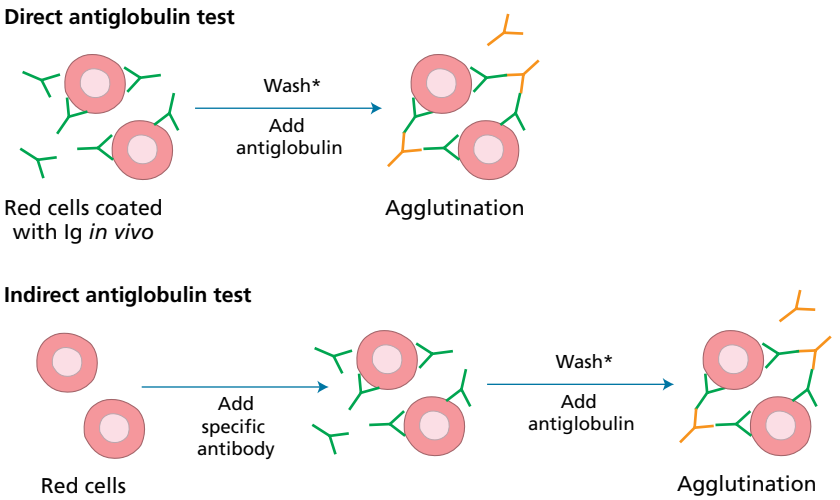

Figure 18.2 Direct and indirect antiglobulin tests. Antiglobulin antibodies are shown in red.*Washing is not required in microcolumn technology.

Inhibition of agglutination

Expected agglutination reactions with known antigens and antibodies can be neutralised by soluble antigens of the appropriate specificity. For example, the saliva of group A secretors inhibits the agglutination of group A cells by anti-A. Hydatid cyst fluid with $P1P^k$ activity can be used to confirm the presence of anti-P1 in sera. Soluble antigens produced by recombinant DNA technology are useful in reference laboratories as aids to sorting out complex serological problems. Certain soluble antigens lend themselves to be more useful than others, either because of the lack of a suitable monospecific serological typing reagent (Dombrock-specific soluble antigen) or the need to inhibit clinically insignificant pan-reactive alloantibodies (CR1 soluble antigen). Additionally, they may be used to inhibit therapeutic monoclonal antibodies, which are increasingly used to treat a range of haematological malignancies, and can interfere with pre-transfusion compatibility testing (for example; anti-CD38).

Haemolysis

After the incubation of red cells with fresh serum, red cell lysis indicates a positive antigen–antibody reaction mediated by IgM complement-fixing antibodies. A pink- or red-coloured supernatant after settling or centrifugation of red-cell–antibody mixtures is an indication of haemolysis.

Adsorption and elution tests

Specific antibodies can be removed from serum by adsorption on red cells carrying the corresponding antigen. Bound antibodies can be subsequently recovered from the washed, sensitised red cells by elution with heat treatment, freeze-thawing, low pH or chloroquine treatment.

Specialised antiglobulin techniques

Flow cytometry and less commonly used enzyme-linked immunosorbent assays (ELISAs) may be used for the estimation of the number of antigen sites on red cells, for the quantitation of anti-D in pregnant women and when more sensitive antiglobulin techniques are required. The essence of these approaches is the use of a labelled antiglobulin: in flow cytometry, which is the most commonly used methodology, fluorescent anti-IgG is used (or antibodies can be directly coupled with a fluorescent dye); in the case of ELISA, enzymes are attached covalently (i.e. conjugated) to the antiglobulin (Figure 18.3).

In these assays, antibody-coated cells or particles are incubated with the labelled antiglobulin reagent. After incubation, excess unbound antiglobulin is washed away and bound labelled antiglobulin is then measured. For flow cytometry, a cytofluorimeter is used to measure fluorescence of each cell. For ELISA, the bound enzyme conjugate is detected through the enzyme's ability to modify its substrate, usually effecting a colour change. The colour intensity of the modified substrate is then measured with a spectrophotometer.

Microcolumn tests (gel and beads)

The principle of microcolumn tests is the separation of agglutinated from non-agglutinated red cells by centrifugation through a miniature filtration column. For blood grouping, red cells are layered on microcolumns impregnated with blood grouping sera; for antibody screening and identification, phenotyped panel red cells are mixed with patients' sera within the incubation chamber of the microcolumn. After centrifugation, agglutinated red cells are retained towards the top of the microcolumn because the agglutinates are trapped by the column matrix,

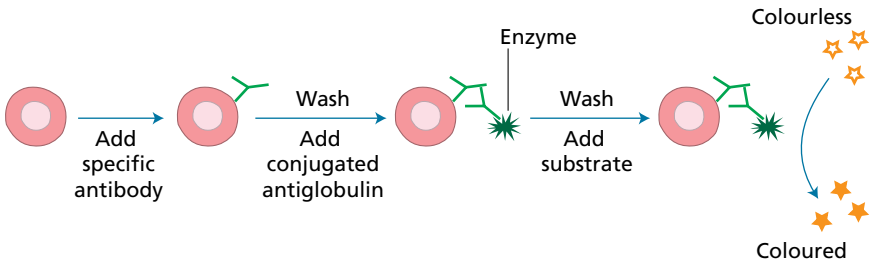

Figure 18.3 Enzyme-linked immunosorbent assay (ELISA) for cell-bound antibodies. Enzyme substrate is represented by open and closed stars, for colourless and coloured derivatives respectively.

whereas unagglutinated red cells form a button at the bottom of the column (Figure 18.4).

Antiglobulin tests can be performed by centrifuging the preincubated mixture of red cells and patient's serum through a column impregnated with anti-IgG or polyspecific antiglobulin. Any red cells coated with IgG will be agglutinated by the free anti-IgG in the matrix of the column, giving a positive result. No washing of sensitised cells is required, as IgG in the serum does not penetrate the column, as it is less dense than the matrix, and so does not neutralise the anti-IgG in the column. The advantages of microcolumn techniques are the ease of reading and reproducibility, and the fact that the tests can be stored for later examination, checking, photocopying or photographing. These systems can be automated and the agglutination results evaluated by image analysis.

Microplate techniques

Semi-automated blood grouping and antibody screening can be performed in microtitre plates, which can also be used for extended phenotyping of red cells, antibody identification and large-scale screening for rare red cells and antibodies.

Figure 18.4 Results of a gel microcolumn test. The subject is group B D-negative. Red cells remaining at the top of the gel represent a positive result; red cells collected at the bottom of the tube represent a negative result.

A single microplate is equivalent to 96 short test tubes and the same basic principles of discrete analysis of agglutination apply. Several commercial microplate-based blood grouping systems are available, using bar codes to identify samples, automated liquid handling of samples and reagents, and plate readers linked to computers for easy and accurate record-keeping.

Solid-phase microplate systems, with antigen or antibody adhered to the surface of the plastic, reduce the variability between tests, inherent in liquid-phase systems when undertaken by different operators, and easily lend themselves to automated reading of results. Blood grouping (e.g. for ABO and D groups) can be accomplished with U-shaped microplate wells coated with the relevant antibody (e.g. anti-A, -B, -D); suspensions of patients' or donors' red cells are added to the wells and then centrifuged. Positive results appear as a carpet of cells coating the bottom of the well. Negative results appear as a tight pinhead of unattached cells in the centre of the well.

In antibody screening and identification by solid-phase, the microplates are coated with a panel of phenotyped red cell ghosts. The wells are incubated with patient serum and LISS, washed and then anti-IgG is added. Anti-IgG will bind to those wells where the patient's IgG antibody has bound to the relevant red cell ghost; bound anti-IgG is then easily detected by adding indicator red cells coated with IgG (e.g. D-positive cells coated with anti-D), followed by brief centrifugation, as described above.

Automated techniques

Fully automated walk-away blood grouping and antibody screening, using microcolumn techniques or microplates, is widely used in transfusion centres, where large numbers of donor samples are tested daily, and in-hospital transfusion laboratories where large numbers of patient samples are tested. In microplate automated systems, test samples are mixed with typing sera or screening cells in individual wells of a special microplate with a terraced surface at the bottom of the well. After incubation and settling of the red cells, agglutination patterns are distinguished either on the basis of light transmission or by image analysis with the aid of a computer-controlled camera.

Blood grouping reagents

In pretransfusion testing, it is essential to determine, as a minimum, the correct ABO and D groups of donors and recipients. To avoid potential fatalities resulting from errors, it is essential that the ABO and D typing reagents have suitable potency and comply with the European Directive on *in vitro* diagnostic devices and the associated Common Technical Specifications and carry the 'CE' mark to show they are in conformance and/or show the equivalent UKCA (UK Conformity Assessed) mark.

IgM monoclonal reagents are generally used for ABO, Rh and K typing. Such reagents are extensively tested prior to release and are generally free of unwanted contaminating antibodies. Blood grouping reagents from polyclonal antisera are more likely to have unwanted antibodies and should have been exhaustively tested with an extensive panel of cells to exclude common and rare specificities before they are issued for routine use.

Antibody screening and identification

For all patients who may require a transfusion, sera should be screened against unpooled group O cells from selected individuals known to carry the following antigens between them: D, C, E, c, e, M, N, S, s, P1, Le^a, Le^b, K, k, Fy^a, Fy^b, Jk^a and Jk^b. Ideally, one cell sample should be R_1R_1 (DCe/DCe), or $R1^WR1$ and the other R_2R_2 (DcE/DcE), so that the Rh antigens all have double dose expression (i.e. homozygous). A minimum double dose expression of Fy^a, Fy^b, Jk^a, Jk^b, S and s should be present on the red cells used for antibody screening and identification. It is generally possible to meet these requirements with two screening cell samples, but if more antigens with double dose expression are required, three cell samples might be needed. Zygosity can be checked by molecular genetic methods.

Antibody screening of patients' sera need only consist of a well-controlled sensitive IAT, using a low ionic strength solution (LISS), commonly with microcolumns. If the antibody screening is positive, antibody identification against a panel of 8–12 fully phenotyped red cells should be performed. For identification, in addition to the IAT, a second sensitive technique (e.g. using enzyme-treated cells, PEG or manual polybrene test), is recommended. Saline tests are not essential for antibody screening or identification and all tests should be performed at 37 °C, as antibodies reacting at lower temperatures only are generally of no clinical importance.

Molecular techniques for blood grouping

It is possible to predict blood group phenotypes from DNA with a high degree of accuracy. This is usually performed when a blood group phenotype is required but a suitable red cell sample is not available. Currently, the most important application is the antenatal determination of fetal blood groups. When a pregnant woman has a blood group antibody with the potential to cause severe HDFN, it is beneficial to determine whether her fetus has the corresponding antigen and consequently, whether it is at risk from HDFN. Hence, molecular typing for D antigen by targeting the *RHD* gene is usually required, but typing for c and K antigens (*RHCE* and *KEL* genes) may sometimes be needed. The standard source material for fetal genotyping is the small quantity of cell-free fetal DNA present in the maternal plasma. In some countries, fetal *RHD* typing is applied to all D-negative pregnant women in order to avoid giving antenatal Rh immunoglobulin prophylaxis unnecessarily when the fetus is predicted D-negative.

Molecular methods for analysis of blood group genes are useful in transfusion-dependent patients, where serological methods are not possible because of the presence of transfused red cells in the patient's blood. Such tests are carried out on DNA isolated from whole blood of the transfused patient. Genotypes can be determined for all clinically important blood group polymorphisms, usually by analysing the relevant single nucleotide variants (SNVs) in blood group genes, such that matched blood can be provided to prevent the patient from making multiple red cell alloantibodies. Another group of patients who would benefit from blood group genotyping are those with autoimmune haemolytic anaemia, whose red cells are coated with immunoglobulin, thus making serological typing difficult.

Several types of tests, ranging from low to high-throughput, are commonly used in blood group genotyping. Most of these are available commercially:

1 Amplification of a portion of a blood group gene by polymerase chain reaction (PCR) to determine its presence. This only applies to *RHD*.

2 PCR amplification of a portion of a gene followed by detection of the restriction fragment length polymorphism with restriction endonuclease enzymes (PCR-RFLP).

3 Selective amplification of a specific allele by the use of an allele-specific primer (allele-specific PCR).

4 PCR amplification of a portion of a gene followed by Sanger sequencing of the amplified product.

5 Allelic discrimination by PCR, incorporating allele-specific fluorescently labelled probes, in which relative quantities of a pair of alleles are measured.

6 Medium/high-throughput methods based on targeting multiple blood group-related SNPs, such as microarray technology, usually involving coloured microbeads, in which numerous polymorphisms can be tested from a relatively small quantity of DNA.

7 Next-generation sequencing (NGS), including whole genome sequencing (WGS), whole exome sequencing (WES) or the targeted sequencing of blood group-related genes. These methods substantially increase sample throughput and the amount of data generated, which enables accurate detection of all known and novel variants in the genes of interest in a single test.

Testing of large numbers of blood donors for multiple blood groups by medium and high-throughput methods is beginning to be introduced in order to establish a database of donors typed for all clinically significant groups. This would be a valuable resource, especially for transfusion-dependent patients, and microarray technology and NGS are now being introduced for such testing.

The ABO system

ABO was the first system to be recognised and remains the most important in transfusion and transplantation. Almost everybody over the age of about 6 months has clinically significant anti-A and/or anti-B in their serum if they lack the corresponding antigens on their red cells. Thus, if we consider the incidence of ABO blood groups in the UK (Table 18.2), transfusions given without regard to ABO would result in a major incompatibility (patient has the antibody and the antigen is on the transfused red cells) about once in every three cases.

Antigens of the ABO system

The blood of the majority of humans can be grouped into six main ABO phenotypes (Table 18.2), although several rare weak variants can be distinguished serologically. The incidence of ABO groups varies very markedly in different parts of the world and in different races.

A_1 and A_2 variants

The distinction between the A_1 and A_2 variants is usually made by using anti-A1, which will agglutinate A_1, but not A_2, red cells. There are several sources of anti-A_1: (i) by absorbing anti-A (from group B people) with A_2 red cells, (ii) in the serum of some A_2 and A_2B persons (Table 18.3), (iii) from a saline extract of the seeds of the hyacinth bean *Dolichos biflorus* and (iv) mouse monoclonal anti-A1. Anti-A1 is not used routinely as it is not necessary to distinguish A_1 from A_2 red cells for most transfusion recipients. There is no specific antibody for A_2 red cells; if anti-A is absorbed with A_1 cells, all the antibody is removed. Group B serum can therefore be thought of as containing two antibodies: anti-A, which agglutinates both A_1 and A_2 red cells, and anti-A1, which agglutinates only A_1 red cells. The anti-A component of group O serum also has both antibodies.

Table 18.2 Incidence of ABO groups in the UK.

Phenotype	Frequency (%)	
O		44.9
A_1	30.8	41.1
A_2	10.3	
B		10.1
A_1B	2.7	3.9
A_2B	1.2	

The difference between the A_1 and A_2 variants is partly quantitative: the red cells of A_1 and A_1B subjects have more A antigen sites than A_2 and A_2B subjects, respectively. For practical purposes, A_2 can be regarded as a weaker form of A. When both A and B antigens are present, there are fewer sites for each than when either is present alone. The practical importance of this lies in the fact that the A antigen of A_2B red cells may give an extremely weak reaction with anti-A, which could be missed in routine grouping tests if reagents of inadequate potency are used. Moreover, if the same person's serum contains anti-A_1 and is tested in the reverse grouping only with A_1 and not A_2 red cells, he/she will be grouped as B. For this reason, potent anti-A reagents reacting with A_2B cells must be used in routine blood grouping. This is now possible with the widespread use of monoclonal antibodies in routine testing.

There is also a qualitative difference between A_1 and A_2, but this must be very subtle because A_2 red cells can absorb all the anti-A

Table 18.3 ABO blood grouping.

Agglutination of test cells with				Agglutination of test serum of			ABO group of test sample	Possible genotype
Anti-A	Anti-A_1	Anti-B	Anti-A, B*	A cells	B cells	O cells		
−	−	−	−	+	+	−	O	*O/O*
+	+	−	+	−	+	−†	A_1	A^1/A^1, A^1/O, A^1/A^2
+	−	−	+	−/+‡	+	−	A_2	A^2/A^2, A^2/O
−	−	+	+	+	−	−	B	*B/B*, *B/O*
+	+	+	+	−	−	−†	A_1B	A^1/B
+	−	+	+	−/+‡	−	−	A_2B	A^2/B

* Anti-A,B (group O serum) is not generally used in routine laboratories.
† Some group A_1 and A_1B individuals may have weak anti-H in their plasma.
‡ Serum from a proportion of A_2 (1–8%) and A_2B (22–35%) individuals contains anti-A1.

from group B serum if the absorption is carried out at 0–4°C for sufficient time. The chemical basis is unresolved, but A-active oligosaccharide structures called type 4A are only present on A_1 cells.

H antigen

Group O cells have no antigens of the ABO system but do possess H antigen, the precursor upon which the products of the *ABO* genes act. The H gene (called *FUT1*) is on chromosome 19, whereas *ABO* is on chromosome 9. The H antigen is present to some extent on almost all red cells, regardless of the ABO group, but the amount of H antigen varies with the ABO group as follows: O > A_2 > A_2B > B > A_1 > A_1B.

Individuals with the rare Bombay phenotype are homozygous for inactive *FUT1* alleles (*h/h*). Their red cells are not agglutinated by anti-A or anti-B, regardless of *ABO* genotype, but are not group O as they are also not agglutinated by anti-H. The serum of Bombay subjects contains potent anti-H, anti-A and anti-B that will only allow transfusion with red cells of the scarce Bombay phenotype.

Distribution of the A, B and H antigens

ABH antigens are often referred to as histo-blood group antigens because they are widely distributed in the body. They are therefore very important in transplantation. They are present on white cells, platelets and epidermal and other tissue cells. They are also present in the plasma, regardless of ABH secretor status, and in the saliva and other secretions of ABH secretors (see later).

ABO variants

Rare ABO variants (often termed ABO subgroups) are usually disclosed because an expected ABO antibody is missing. A sample typed as group O that has anti-B but no anti-A will usually prove to be a weak A variant. The presence of weak A or B antigens can be demonstrated either by using potent monoclonal antisera or by adsorption and elution. A variety of A and B variant phenotypes exist with the symbols A_3, A_x, A_{end}, A_m A_{el}, B_3, B_x, B_m and B_{el}. All are rare, and are usually recognised by their variable reactions with anti-A and/or anti-A,B reagents, and arise from mutations in the coding or regulatory regions of the *ABO* gene.

Acquired weakening of the A antigen can also occur in acute myeloid leukaemia. The A antigen may revert to almost normal in remission. Similar weakening of B, H and I antigens has also been described.

B-like antigens may be acquired by group A individuals who are suffering from bowel infections, usually associated with carcinoma or strictures of the large bowel. Red cells with an acquired B antigen are agglutinated by some anti-B, including some monoclonal anti-B, but not by the patient's own anti-B. Bacterial deacetylases convert *N*-acetylgalactosamine, the immunodominant sugar of the A antigen, into galactosamine, a structure similar to galactose, the immunodominant sugar of the B antigen.

Antibodies of the ABO system

Anti-A, anti-B and anti-A,B

It is likely that 'naturally occurring' ABO antibodies arise in response to A- and B-like antigens present on bacterial, viral or animal molecules. Titres of ABO antibodies vary considerably with age, reaching a peak in young adults and then declining in old age.

Sera taken from people over the age of about 6 months that do not contain the expected A and B antibodies (Table 18.3) are very rare. They should always be investigated thoroughly; often, some interesting explanation will be found, for example a rare subgroup of A, a blood group chimera or congenital absence of IgM.

Anti-A and anti-B have a wide thermal range. Although they are active at 37°C, they react better at lower temperatures. ABO antibodies always have some IgM component and, in group A and B persons, they are almost entirely IgM. Antibodies from group O individuals, even before immunisation, usually have some IgG anti-A,B, an antibody that cross-reacts with both A and B structures.

Following immunisation with red cells or blood group substances, the thermal characteristics of the antibodies change, but group A and B subjects continue to produce antibodies that are mainly IgM. Most group O persons, however, will produce IgG as readily as IgM anti-A,B. Consequently, mothers of children with ABO HDFN are almost always group O. Immune anti-A,B are mainly IgG2, which does not cause HDFN because there are no Fc receptors for IgG2 on the cells of the mononuclear phagocyte system. When the maternal serum contains potent IgG1 and/or IgG3 ABO antibodies, HDFN may occur, although this is comparatively milder than HDFN caused by antibodies to Rh blood group antigens (see Chapter 19). Some IgA anti-A or anti-B may be produced following immunisation with A or B substances.

Group O as a universal donor

Group O red cells can be transfused to A, B or AB recipients and group O donors were formerly, and inappropriately, called 'universal donors'. Group O donors have anti-A, anti-B and anti-A,B in their plasma, which will react with the recipient's A or B cells. Normally, if group A, B or AB recipients are transfused with a relatively small number of group O units of whole blood, the anti-A or anti-B that is transfused will be diluted out and neutralised by the plasma of the adult recipient, especially as red cells in additive solution are now used. The use of whole blood in trauma is becoming increasingly popular, firstly in use by the military, it is now being used in some civilian trauma and pre-hospital emergency settings. Components used are typically a combination of leucodepleted,

platelet poor group O RBC and Gp O plasma of low ABO haemolysin titre to mitigate against any ABO antibody-mediated haemolysis.

There is usually a shortage of group O blood, and not infrequently a surplus of group A blood. In the vast majority of cases, including emergencies, there is enough time to perform a rapid ABO group on the patient's cells, which will allow the transfusion of group-specific blood. If there is no time to do an ABO group before transfusion, group O red cells in optimal additive solution, devoid of plasma, should be given until the patient's blood group is known. The practice of transfusing group O platelets to non-O patients should be discouraged as the dose of adult platelets will contain approximately 300 mL of donor plasma, unless part of it has been replaced by platelet-additive solution. Group O fresh-frozen plasma and cryoprecipitate should only be given to group O recipients.

Anti-A1

Anti-A1, reactive at room temperature (18–22 °C), can be found in the serum of 1–8% of group A_2 and 22–35% of group A_2 B persons. Most of these antibodies are not of clinical importance because they do not agglutinate A_1 red cells at 30 °C and above. Very rarely, anti-A1 able to react at 37 °C may lead to significant destruction of A_1 red cells *in vivo*. Group A or group O red cells, crossmatch compatible by IAT, should be provided in these rare instances.

Anti-H

Several forms of anti-H exist:

1 Clinically significant 'true' anti-H occurs in the serum of very rare individuals with the Bombay phenotype (O_h) blood. It is active at 37 °C and only O_h blood may be transfused.

2 Anti-HI, commonly found in the serum of group A_1, B and A_1B persons, reacts much more strongly with adult than with cord red cells. Anti-HI does not usually agglutinate O cells above 30 °C. Very occasionally, anti-HI may cause rapid destruction of at least some of the transfused O red cells *in vivo*. However, these antibodies will not interfere with red cell survival if ABO identical units, i.e. A_1, B or A_1B units, are transfused to A_1, B or A_1B recipients, respectively.

ABH secretor status

About 80% of Caucasian populations, ABH secretors, have H antigen, plus A or B according to their ABO genotype, in a water-soluble form in their body secretions. The remaining 20% are non-secretors.

Biochemistry and biosynthesis of ABH antigens

ABH antigens

A, B and H antigens on red cell membranes (Figure 18.1) are predominantly glycoproteins, the majority being on the N-glycans of the anion exchanger (band 3) and the glucose transporter (GLUT1). ABH antigens on red cells are also expressed on glycosphingolipids. Soluble ABH antigens are glycoproteins. Differences in the terminal sugars of the glycoproteins and glycolipids determine the specificity of these antigens: L-fucose (Fuc) for H, L-fucose plus *N*-acetyl-D-galactosamine (GalNAc) for A and L-fucose plus D-galactose (Gal) for B.

Two major types of carbohydrate chain endings serve as acceptors for the fucosyltransferases that synthesise H antigen: type 1 and type 2 chains have Gal joined to *N*-acetylglucosamine (GlcNAc) through 1 → 3 and 1 → 4 linkages, respectively. A- and B-transferases transfer GalNAc and Gal, respectively, from their donor substrates UDP-GalNAc and UDP-Gal to the terminal galactosyl residue of type 1 H and type 2 H, creating A and B epitopes and masking H specificity (Figure 18.5).

Secretory glycoproteins possess both type 1 and type 2 linkages, whereas red cells synthesise type 2 chains only. Other chains, called type 3 and 4, are also present in low numbers on red cells, but probably only on glycolipids.

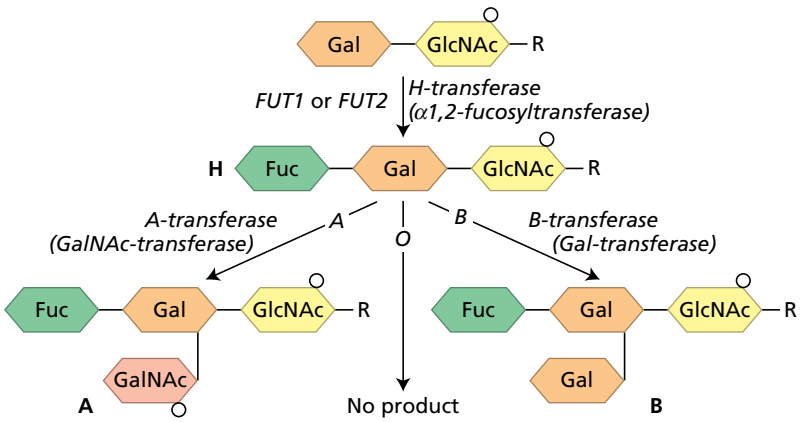

Figure 18.5 Biosynthetic pathway of H antigen from its precursor, and of A and B antigens from H. In group O subjects, H remains unconverted in the absence of *A* or *B* gene products. R, remainder of molecule.

ABO genes

ABO is located on the long arm of chromosome 9, comprises seven exons and encodes the A and B glycosyltransferases. Products of the *A* and *B* alleles differ by four amino acids encoded by exon 7, two of which determine whether the enzyme has GalNAc-transferase (A) or Gal-transferase (B) activity.

The majority of *O* alleles (called O^1) resemble *A*, but have a single-base deletion in exon 6, which creates a shift in the reading frame, truncating any putative polypeptide. About 3% of *O* alleles (called O^2) have a single-nucleotide polymorphism (SNP) that changes one of the vital amino acids in the catalytic site, inactivating the enzyme.

The A^2 allele has a single-base deletion immediately before the usual termination codon, creating a reading frameshift and abolition of this stop codon. This produces an A-transferase with 21 extraneous amino acids on its C-terminus, reducing its efficiency as a GalNAc-transferase.

H genes

At least two genes, *FUT1* and *FUT2*, on chromosome 19, are responsible for production of H antigen. Both encode α1,2-fucosyltransferases that catalyse the transfer of fucose to the terminal galactose of the H precursor chain (Figure 18.5). *FUT1* is active in mesodermally derived tissues, including haemopoietic tissues, and is responsible for H expression on red cells. Homozygosity for inactivating mutations in *FUT1* gives rise to O_h (Bombay) and related phenotypes. *FUT2* is responsible for the expression of H antigen in endodermally derived tissues, including secretions, and hence is the gene responsible for ABH secretion. Secretors are homozygous or heterozygous for an active *FUT2*; non-secretors are homozygous for an inactive allele usually containing a nonsense mutation.

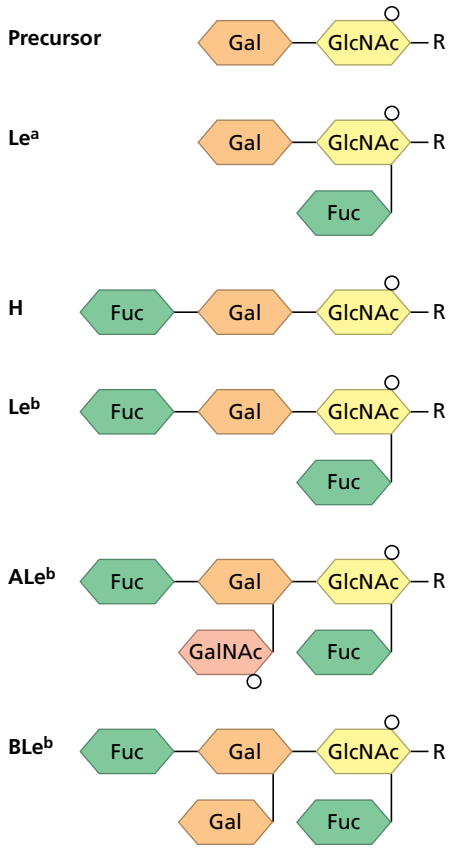

Figure 18.6 Diagrammatic representation of H and Lewis antigens in plasma and secretions. Le^a requires the action of the Lewis α1,4-fucosyltransferase (encoded by *FUT3*), H the action of the H α1,2-fucosyltransferase (encoded by *FUT2*), Le^b the action of both Lewis and H fucosyltransferases, and ALe^b and BLe^b the action of Lewis and H fucosyltransferases and the A or B glycosyltransferases.

The Lewis system

Lewis antigens and their biosynthesis

Lewis is primarily a system of soluble antigens present in secretions and in plasma: Lewis antigens on red cells are adsorbed passively from the plasma, and plasma is needed to maintain Lewis antigens on the red cells. There are two Lewis antigens: Le^a and Le^b. Expression of either requires the presence of an active Lewis gene, but Lewis phenotypes are also governed by the gene controlling H secretion (*FUT2*). Lewis antigens in saliva and plasma are glycoproteins and glycolipids, respectively.

The Lewis gene, *FUT3*, encodes an α1,4-fucosyltransferase that catalyses the addition of L-fucose in 1→4 linkage to the subterminal GlcNAc of type 1 chains (Figure 18.6). Since red cells have only type 2 chains, they cannot make Lewis antigens. If the type 1 chain has been unmodified, the Lewis-transferase makes Le^a antigen, whereas if the secretor α1,2-fucosyltransferase has converted the type 1 chains to type 1 H, Le^b is produced. In a white population, 75% have active Lewis and secretor genes and hence Le(a−b+) red cells; 20% have an active *Lewis* gene but are H non-secretors and have Le(a+b−) red cells; 5% are homozygous for *Lewis* genes with inactivating mutations and are Le(a−b−) (Table 18.4). Le(a+b−) and Le(a−b+) red cells incubated in Le(a−b−) plasma shed their Lewis antigens into the plasma. Similarly, if Le(a+b−) or Le(a−b+) red cells are transfused into an Le(a−b−) recipient, the transfused cells will gradually lose their Lewis antigens and will group as Le(a−b−) within 1 week of transfusion.

Development of Lewis antigens on red cells

The Lewis antigens are poorly developed at birth and red cells from cord blood are usually Le(a−b−). Thereafter, Le^a develops first, followed by Le^b if the relevant *Lewis* and secretor genes are present. Red cells of children between the ages of 6 months and

Table 18.4 The Lewis system and secretion of ABH.

Genotype		Antigens in saliva			Plasma/red cells*	
FUT2 (secretor)	**FUT3 (Lewis)**	**Le^a**	**Le^b**	**ABH**	**Le^a**	**Le^b**
Se/Se or *Se/se*	*Le/Le* or *Le/le*	+	++	++	−	++
Se/Se or *Se/se*	*le/le*	−	−	++	−	−
se/se	*Le/Le* or *Le/le*	+++	−	−	++	−
se/se	*le/le*	−	−	−	−	−

Le, active allele at *FUT3* locus; *le*, inactive allele; *Se*, active allele at *FUT2* locus; *se*, inactive allele.
* Le^a and Le^b on red cells are passively adsorbed from plasma.

4 years often type as Le(a+b+) if they are destined to become Le(a−b+). The definitive adult Lewis phenotype may not be reached until the age of 4–5 years.

Lewis antibodies

Lewis antibodies are generally made only by Le(a–b–) individuals. Anti-Le^a occurs fairly frequently. The incidence of Le(a−b−) is much higher in people of African and Southeast Asian origin than in Europeans, and Lewis antibodies may be found in up to 10% of random serum samples from black people.

Anti-Le^b commonly accompanies anti-Le^a. Pure anti-Le^b is uncommon.

Lewis antibodies are predominantly IgM. They usually agglutinate the appropriate cells at 20 °C. All Lewis antibodies that react at 37 °C will bind complement and may lyse antigen-positive cells. Lewis antibodies do not agglutinate red cells in saline at 37 °C, but can be detected with anti-complement in an indirect antiglobulin test (IAT).

Anti-Le^a active at 37 °C is usually more haemolytic than anti-Le^b, and some anti-Le^{a+b} can be very potent, leading to intravascular red cell destruction in the initial stages of transfusion. All Lewis antibodies active at 37 °C lead to two-component survival curves of transfused incompatible red cells, i.e. the first cells are destroyed at an accelerated rate and the remainder, which have lost their adsorbed Lewis antigens into the recipient's Le(a–b–) plasma, will have a normal survival.

For patients with Lewis antibodies reacting at 37 °C, ABO-identical red cells, compatible in an IAT cross-match at 37 °C, should be transfused. It is important to choose ABO-identical cells because group A, B and AB cells carry fewer Lewis antigens than group O cells, which are the cells routinely used in antibody screening. The provision of pre-typed Le(a−b−) blood for patients with Lewis antibodies is not necessary, as it is always easy to find ABO identical, cross-match-compatible red cells at 37 °C.

Lewis antibodies do not cause HDFN as they are IgM and, moreover, newborn infants have Le(a−b−) red cells.

P1, P^k and P blood group antigens and antibodies

P1 antigen is present on red cells of about 75% of Caucasians though P1 frequency varies in different populations. P1 is weakly expressed at birth and its strength varies considerably in adults. For this reason, identification of anti-P1 can be difficult, as panel cells will have varying expression of the antigen.

Anti-P1 is naturally occurring and commonly found in the serum of P1-negative individuals. Unlike anti-A and anti-B, anti-P1 rarely causes transfusion reactions because it is usually not reactive above 30 °C.

Anti-PP1P^k and anti-P invariably occur in the sera of the very rare individuals with p and P^k phenotypes, respectively. Anti-PP1P^k reacts with all red cells except those of the p phenotype; anti-P reacts with all red cells except those of the P^k and p phenotypes. They are usually strong IgM antibodies, often lytic at 37 °C, and can cause severe haemolytic transfusion reactions. If p or P^k patients need transfusion, rare allogeneic red cells will need to be sourced or pre-deposit autologous donation may need to be considered. Occasionally, IgG anti-P or anti-PP1P^k have been associated with spontaneous early abortion.

The biphasic Donath–Landsteiner autoantibody, found in the sera of patients suffering from paroxysmal cold haemoglobinuria, is always IgG and usually has anti-P specificity.

I and i antigens and antibodies

I and i antigens are carbohydrates and are on the interior structures of the complex oligosaccharides that carry ABO, H and Lewis antigens; i is the biosynthetic precursor of I. The i antigen represents linear structures that are converted to I-active branched structures by the transferase coded by the *I* gene. This enzyme is not active in neonates. Consequently, red cells of most adults are agglutinated strongly by anti-I and only weakly by anti-i, whereas red cells from cord blood give the opposite result. Adults who are homozygous for rare inactivating

mutations in the *I* gene have the adult i phenotype; their red cells react weakly or negative with anti-I and strongly with anti-i, and their serum contains anti-I.

Autoanti-I active at 20 °C, but not at 30 °C, occurs in a variety of disorders and after blood transfusions. These patients' red cells give a negative direct antiglobulin test. A transient increase in strength, titre and thermal range of anti-I regularly occurs after infections with *Mycoplasma pneumoniae* and occasionally leads to acute haemolysis; red cells of such patients give a positive DAT, as do those of patients suffering from chronic cold haemagglutinin disease (CHAD). In CHAD, the autoantibody is nearly always monoclonal and usually has anti-I specificity.

Autoanti-i is found transitorily in many patients suffering from infectious mononucleosis. Very occasionally, the titre and thermal range of this antibody may lead to acute haemolysis. Autoanti-i may occasionally be the antibody specificity in chronic CHAD. Such patients often have an underlying lymphoma.

The Rh system

Rh is the second most important blood group system in blood transfusion. Anti-D is formed readily when D-positive blood is transfused to a D-negative person. The Rh system contains a total of 56 antigens, but D is the most important, owing to its high immunogenicity and ability to cause severe HDFN and HTRs.

Rh antigens

Alloanti-D was initially considered to be identical to antibodies produced in rabbits and guinea pigs immunised with rhesus monkey red cells. Later animal anti-Rh was shown to detect a different determinant renamed LW. LW is now known to be intercellular adhesion molecule-4 (ICAM-4). A remnant of the original name, rhesus, remains in the name of the blood group system, Rh. For clinical purposes, individuals are often called Rh-positive if they have the D antigen and Rh-negative if they lack D. Approximately 85% of Caucasians, 95% of Africans and over 99% of eastern Asians are D-positive.

Two pairs of antithetical antigens, C (RH2) and c (RH4), and E (RH3) and e (RH5), are closely associated with D genetically. There is no antithetical antigen to D, so d represents the absence of D. From serological analyses, D, C/c and E/e behave like the products of three closely linked genes and the eight Rh haplotypes are often written as *DCe*, *DcE*, *dce*, etc (shorthand, R_1, R_2, *r*, respectively) (Table 18.5).

Molecular genetics has shown that there are only two *RH* genes, one encoding D, *RHD,* the other encoding the Cc and Ee antigens, *RHCE,* but as the Cc and Ee polymorphisms are determined by separate regions of a single gene, the DCE terminology is still suitable for understanding Rh at most levels. The approximate frequencies of the Rh gene complexes in three populations are shown in Table 12.5. Genotype frequencies vary considerably in different parts of the world: e.g. *dce/dce* (Rh-negative) varies from about 35% in Basques to 0.3% in Japanese and Chinese.

Table 18.5 Eight Rh haplotypes and their frequencies in English, Nigerian and Hong Kong Chinese populations.

Haplotype		Frequencies (%)		
CDE	**Rh-Hr**	**English**	**Nigerian**	**Chinese**
DCe	R_1	42	6	73
dce	*r*	39	20	2
DcE	R_2	14	12	19
Dce	R_0	3	59	3
dcE	r''	1	Very rare	Very rare
dCe	r'	1	3	2
DCE	R_Z	Rare	Very rare	Rare
dCE	r^y	Very rare	Very rare	Rare

Results of testing, with anti-D, -C, -c, -E and –e, red cells from 2000 English donors, 274 Yoruba of Nigeria and 4648 Cantonese from Hong Kong.

Probable Rh genotype

When a person's Rh phenotype is known, the probable genotype can be discerned and its likelihood calculated from known genotype frequencies within the same population. When probable genotype determinations are carried out, it is very important that the ethnic origin of the person is known; figures for one population will not apply to people of other populations. For example, in white populations, *dce* is 15 times more common than *Dce*, whereas in African populations *Dce* has a slightly higher frequency than *dce*. Consequently, the phenotype D + C + c + E − e+ represents a probable genotype of *DCe*/*dce* in a white person, but of *DCe*/*Dce* in a black person.

Molecular genetics of Rh

Rh genes and proteins

Rh antigens are encoded by two closely linked genes with 92% sequence homology. *RHD* encodes the D antigen and *RHCE* the Cc and Ee antigens. Each consists of 10 exons and, unusually for homologous genes, the two genes are in opposite orientations on the chromosome (Figure 18.7). Each gene encodes a 416-amino-acid polypeptide of 30–32 kDa that is palmitoylated but not glycosylated. The polypeptides encoded by *RHD* and *RHCE* differ by 31–35 amino acids, depending on the *RHCE* genotype. The Rh polypeptides traverse the lipid bilayer 12 times, with both termini in the cytoplasm and six extracellular loops that provide the putative sites for antigenic activity (Figure 18.7). Rh antigen activity is very dependent on

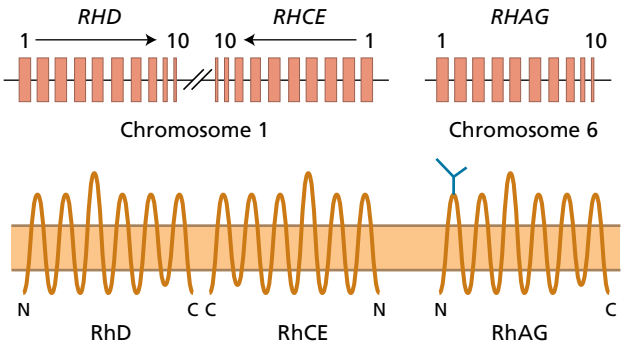

Figure 18.7 Rh and related genes (in pink) and the polypeptides they encode (in orange), showing the 10 exons of *RHD* and *RHCE* in reverse orientation on chromosome 1 and of *RHAG* on chromosome 6, and the RhD and RhCE polypeptides and RhAG glycoprotein crossing the membrane 12 times.

the conformation of the proteins within the membrane and may involve interactions between two or more of the extracellular loops.

D, C/c and E/e polymorphisms

In white people, the D-negative phenotype almost always results from homozygosity for a complete deletion of the *RHD* gene. D-negative represents the absence of the RhD protein from the membrane. D-positive people may be homozygous or hemizygous for *RHD*. However, most D-negative black Africans have an inactive *RHD*, called the *RHD* pseudogene (*RHDψ*).

The Cc polymorphism is associated with three or four amino acid substitutions encoded by exons 1 and 2 of *RHCE*, although the definitive change is Ser103 for C and Pro103 for c in the second extracellular loop of the RhCE protein. E and e are associated with Pro226 and Ala226, respectively, in the fourth extracellular loop.

The band 3/Rh molecular macrocomplexes

The two Rh proteins are closely associated in the red cell membrane with the Rh-associated glycoprotein (RhAG). RhAG has a similar conformation, spanning the membrane 12 times, but is glycosylated (Figure 18.7). The complex of Rh proteins and RhAG is part of a membrane macrocomplex, with band 3 (the anion exchanger and Diego blood group antigen) at its core, and also containing the LW glycoprotein (ICAM-4), CD47 and glycophorins (GP)A and B. This macrocomplex is attached to the cytoskeleton, primarily through ankyrin and protein 4.2 (Figure 18.1). Another macrocomplex containing band 3 and the Rh proteins also contains the Kell, Kx and Duffy proteins, plus GPC (Gerbich), which links the complex to the cytoskeleton.

The Rh proteins are only present on erythroid cells, but RhAG is more widely distributed.

Variants of D

Most D-negative individuals lack the whole RhD protein from their red cells and, when immunised by D-positive red cells, can make antibodies to an array of epitopes on the external loops of the RhD protein. About 30 D epitopes have been defined with monoclonal antibodies. There are numerous variants of D. These used to be divided into two types, weak D (formerly D^u) and partial D, though these terms are not adequately defined and of little value clinically. The usual understanding is weak D red cells express all epitopes of D at a low level and individuals with weak D phenotype cannot make anti-D, whereas partial D red cells lack some epitopes of D and so individuals with a partial D phenotype, if immunised by a normal complete D antigen, can make antibodies to the D epitopes they lack. These antibodies behave as anti-D in most circumstances. However, things are not that simple and some phenotypes named weak D have subsequently been found in individuals who have made anti-D, and in addition, some so-called partial D antigens are expressed very weakly. So, the terms weak D and partial D should be abolished and the abnormal D antigens all included in the term D variant.

The most important D variant, from a transfusion perspective, is DVI ('D six'). It is not the most common, but lacks most of the D epitopes and is, therefore, the D variant most commonly associated with the production of anti-D. In the UK, it is recommended that anti-D reagents for typing patients should not detect DVI. Consequently, DVI patients will be typed as D-negative and transfused with D-negative red cells and DVI pregnant women will be given anti-D prophylaxis. Ideally, one of the anti-D reagents used for typing donor red cells should detect DVI; hence DVI donors will be typed as D-positive. This strategy means that DVI individuals should be considered D-negative patients, but D-positive donors. Weak D types 1, 2, and 3 are three of the most common D variants and are almost never associated with anti-D production.

In view of the high quality of monoclonal anti-D in the UK, the recommended method for D typing of patients requires direct agglutination tests, in duplicate, with potent IgM monoclonal anti-D reagents. An antiglobulin test is no longer required. Patients whose red cells appear D-positive, but give weak or inconsistent reactions, suggestive of a D variant, are treated as D-positive and transfused with D-positive blood unless they are females of childbearing potential or transfusion dependent. In those cases, they are treated as D-negative and transfused with D-negative blood unless they are shown to have the weak D type 1, 2 or 3. This strategy is designed to conserve valuable stocks of D-negative red cells for patients who really need them.

Similarly, donors are not typed any longer for D by an antiglobulin test because it is unlikely that transfusion of very weak D red cells to a D-negative patient will result in immunisation of the patient. In some countries, however, the antiglobulin test for D typing of donors is still required and in some countries, a molecular test is mandatory for confirming D-negative status of donors.

Molecular basis of D variants

D variants result either from missense mutations in *RHD*, encoding single amino acid changes or from an *RHD* in which a segment has been replaced by the equivalent segment of an *RHCE* gene due to unequal crossing over or gene conversion. For example, the most common form of DVI is produced by an *RHD–CE–D* hybrid gene in which exons four to six have the nucleotide sequence of *RHCE*, and external loops 3 and 4 of the encoded hybrid protein have the amino acid sequence of an RhCe protein. Hence, a protein is produced with a sequence similar enough to RhD to be stable in the membrane, but different enough to lack many epitopes of D. A variety of names have been used to denote the different types of D variants, for example DIII, DVI, DFR, DAR and DBT, which can be distinguished by sophisticated serological techniques, and weak D types 1–145, which can only be distinguished by molecular techniques.

Antibodies of the Rh system

Naturally occurring antibodies

Generally, Rh antibodies are only produced following immunisation with red cells. However, anti-E is often naturally occurring; about half may occur without a history of pregnancy or transfusion. Rarely, naturally occurring anti-D and anti-C^W are found. All such naturally occurring Rh antibodies react optimally with enzyme-treated cells; if they react at 37 °C by IAT, they are clinically significant.

Immune antibodies

The clinical importance of the Rh system lies in the readiness with which anti-D arises in D-negative subjects after stimulation with D-positive red cells by pregnancy or transfusion. Prophylaxis against D immunisation with anti-D immunoglobulin in pregnancy (see below) has led to a significant decrease in the incidence of anti-D, but it still remains the most common immune antibody of clinical relevance detected in a routine blood transfusion laboratory. D is considerably more immunogenic than the other Rh antigens, which have the following order of immunogenicity: c > E > e > C.

About 20–30% of anti-D sera also appear to contain anti-C. Usually, this anti-C is not a separable antibody and is probably more correctly called anti-G. About 1–2% of anti-D sera also contain anti-E. Anti-C (and anti-G) in the absence of anti-D is very uncommon.

The incidence of other Rh antibodies is much lower, but together they are more common than anti-K (Kell system), which is the most immunogenic antigen after D. In routine screening, pure anti-E is the most common, followed by anti-c, although anti-c is a more common cause of HDFN, which can be severe. This is probably because many anti-E are weak, naturally occurring antibodies. Anti-e, like anti-C, is rare.

The vast majority of Rh antibodies are IgG and do not fix complement. Anti-D may occasionally be partly IgA. IgM anti-D is very rare.

D immunisation and immunoprophylaxis

The D antigen is very immunogenic; over 80% of D-negative recipients of a whole unit of D-positive red cells make anti-D and 17% of D-negative women will be immunised by one pregnancy with a D-positive offspring.

Prediction of fetal RhD type by molecular methods

Knowledge of the molecular basis for D-negative phenotypes has made it possible to devise tests for predicting fetal D type from fetal DNA. This is valuable in determining whether the fetus of a woman with anti-D is at risk from HDFN. Most methods involve PCR-based tests that detect the presence or absence of *RHD*, but it is important to test for more than one region of *RHD*, so that hybrid genes responsible for partial D antigens do not give a false result, and to test for the *RHD* pseudogene (*RHDψ*), so that this does not give rise to a false-positive result.

Although fetal DNA can be obtained by amniocentesis, this procedure is associated with a significant risk of fetal loss and fetal-maternal haemorrhage. The best source is the small quantities of free fetal DNA present in maternal plasma. This noninvasive form of fetal D typing is now provided for pregnant women with significant levels of anti-D as a routine service in the UK and other parts of Europe and should be considered the standard of care in these patients. This technology is now also applied to high-throughput non-invasive prenatal testing (NIPT) for fetal *RHD* genotype of D-negative pregnant women, to determine if anti-D prophylaxis is required. This approach means that only the D-negative women carrying a D-positive fetus will receive anti-D and those carrying a D-negative fetus will not have anti-D administered unnecessarily. Fetal D testing of all D-negative pregnant women is provided as a routine service in the UK and several European countries. When this testing is not available, it is common practice to offer antenatal anti-D prophylaxis to all D-negative pregnant women. In a predominantly white population, about 40% of these women will have a D-negative fetus and therefore receive the treatment unnecessarily.

The MNS system

Antigens of the MNS system

M and N are inherited as codominant Mendelian traits, giving rise to three common genotypes *M*/*M*, *M*/*N* and *N*/*N*. The *Ss* locus, which is closely linked to *MN*, also consists of two codominant alleles in Europeans and Asians, producing S or s antigens. In northern Europeans, haplotype frequencies are as follows: *MS*, 25%; *Ms*, 28%; *NS*, 8%; *Ns*, 39%. The system also contains

many variants. About 2% of black West Africans and 1.5% of African-Americans are S−s− and most of these lack the U antigen that is present when either S or s is expressed.

The MN antigens are carried on glycophorin A (GPA), which is encoded by the *GYPA* gene on chromosome 4. There are about 1 million molecules of GPA per cell, yet their absence in the rare En(a−) phenotype does not affect red cell function or survival. The negative charge of the red cells is mainly due to the ionised COOH groups of sialic acid (neuraminic acid), which is mostly carried on the oligosaccharides of GPA and can be removed with neuraminidase. En(a−) cells therefore have a reduced negative charge and behave serologically as if they have been treated with neuraminidase. GPB carries the S and s determinants. GPB is encoded by *GYPB*, which is closely linked and homologous to *GYPA*. S−s−U− red cells lack GPB. GPA and GPB are exploited as receptors by the malaria parasite *Plasmodium falciparum*.

Antibodies of the MNS system

Anti-M is uncommon and is usually naturally occurring, but can be immune and, very rarely, causes HDFN. Anti-N is also rare and is nearly always cold-reactive IgM. Useful anti-N lectin can be prepared from the seeds of *Vicia graminea*. Anti-S, the rarer anti-s and anti-U are usually immune, IgG and can cause HDFN. They have also been implicated in HTRs. Anti-U only occurs in S−s− black people and reacts with all cells that have the S or s antigens and up to 50% of cells that are S−s− and have a variant U antigen. Finding compatible blood for a patient with anti-U can prove difficult, especially in a predominantly White donor population.

The Kell blood group system

The Kell blood group system consists of 38 antigens, including one triplet and five pairs of allelic antigens – K and k; Kp^a, Kp^b and Kp^c; Js^a and Js^b; K11 and K17; K14 and K24; KHIZ and KHOZ – all of which represent amino acid substitutions in the Kell glycoprotein. Three sets of antigens are clinically important: K/k; $Kp^a/Kp^b/Kp^c$ and Js^a/Js^b.

In white Europeans, the incidence of the K/k phenotypes, the most important clinically, is as follows: K+k−, 0.2%; K+k+, 8.7%; K−k+, 91.1%. K is rare in non-white populations. Js^a is present in about 20% of black people, but is extremely rare in other ethnic groups. K_0, a very rare null phenotype of the Kell system in which no Kell antigens are expressed, results from homozygosity, or compound heterozygosity, for a variety of inactivating mutations in the *KEL* gene.

The Kell antigens are located on a glycoprotein that crosses the cell membrane once, with a large glycosylated C-terminal extracellular domain, maintained in folded conformation by multiple disulfide bonds. Reduction of these bonds by dithiothreitol (DTT) results in loss of expression of all Kell antigens, leading to artificial K_0 cells with elevated expression of Kx.

Anti-K is an important antibody in white populations; it is nearly always immune, IgG and complement-binding. It causes severe HTRs and HDFN. K stimulates the formation of anti-K in about 10% of K-negative people given one unit of K-positive blood. About 0.1% of all cases of HDFN are caused by anti-K; most of the mothers will have had previous blood transfusions. HDFN caused by anti-K differs from Rh HDFN in that anti-K causes fetal anaemia by suppression of erythropoiesis, rather than immune destruction of mature fetal erythrocytes. Kell antigens are expressed by erythroid cells at a very early stage of erythropoiesis and anti-K would facilitate destruction of K+ early erythroid progenitors, before they become haemoglobinised. Anti-k is very rare, reacting with 99.8% of random blood samples. It is always immune and has been implicated in HDFN. Most other Kell system antibodies are rare, best detected by the IAT and are usually clinically significant.

The Kell glycoprotein is linked by a single disulfide bond to the Kx protein, produced by an X-linked gene, *XK*. Absence of Kx, resulting from hemizygosity in males of a deletion of *XK* or of *XK* containing inactivating mutations, gives rise to the McLeod syndrome, in which there is no expression of Kx antigen, weak expression of all Kell system antigens and a neuroacanthocytosis that is usually characterised by late-onset muscular, neurological and psychiatric disorders, plus about 30% red cells with acanthocytosis and associated haemolytic anaemia. In some cases, an X chromosome deletion is large enough to encompass *XK* and *CYBB*, a gene for a subunit of flavocytochrome b_{558}, which leads to McLeod syndrome and chronic granulomatous disease (CGD). However, the majority of CGD patients have normal Kell antigens and Kx. If boys with McLeod syndrome and CGD are transfused, they are able to make anti-Kx, which can cause severe HTRs, most often needing to resort to the International Rare Donor Panel for compatible blood.

Other blood group systems

Fy^a and Fy^b antigens of the Duffy system represent a single amino acid substitution in the extracellular N-terminal domain of the Duffy glycoprotein. Their incidence in the UK is as follows: Fy(a+b−), 20%; Fy(a+b+), 46%; Fy(a−b+), 34%. About 70% of African–Americans and close to 100% of West Africans are Fy(a−b−). Anti-Fy^a is not infrequent. It is IgG, often complement-fixing and can cause HTRs, but seldom HDFN. It is best detected by IAT and does not react with red cells treated with the proteases papain and ficin. Anti-Fy^b is very rare and is always immune.

Kidd has two alleles, Jk^a and Jk^b, which represent a single amino acid change in the Kidd glycoprotein. Phenotype frequencies in the UK population are as follows: Jk(a+b−), 25%; Jk(a+b+), 50%; Jk(a−b+), 25%. The Kidd null phenotype, Jk(a−b−) is very rare in most populations but reaches an incidence of greater than 1% in Polynesians. Anti-Jk^a is uncommon and anti-Jk^b is even rarer, but they may both cause severe

transfusion reactions and, to a lesser extent, HDFN. Kidd antibodies have often been implicated in delayed HTRs; they are IgG and predominantly complement-fixing up to C3b, but may be difficult to detect because they tend to disappear and then reappear promptly in an anamnestic response. The antiglobulin test is the best method for detection; reactions are enhanced if the cells are protease-treated or if fresh serum is added as a source of complement.

Lutheran is a complex system and includes the antigens Lu^a and Lu^b, which represent a single amino acid substitution in the Lutheran glycoprotein. Phenotype incidence in the UK is as follows: Lu(a+b−), 0.1%; Lu(a+b+), 7.5%; Lu(a−b+), 92.4%. The extremely rare Lu_{null} phenotype, in which no Lutheran antigens are expressed, results from homozygosity for an inactive Lutheran gene; anti-Lu3 may be produced. Lutheran antibodies are uncommon and are not generally clinically significant, although anti-Lu^b may have caused mild delayed HTRs. Lu^a may be omitted from antibody screening cells.

The Diego system antigens represent single amino acid substitutions in band 3, the red cell anion exchanger. Di^a, is very rare in white and black people, but relatively common in certain populations, with frequencies varying between 1% in Japanese and 50% in some native South Americans. Anti-Di^a and anti-Di^b are immune and rare but can cause HDFN. Wr^a has a frequency of about 0.1%. Naturally occurring anti-Wr^a is present in approximately 1% of blood donors. Anti-Wr^a is often found in the serum of patients who have made other antibodies or who are suffering from autoimmune haemolytic anaemia. Very rarely, anti-Wr^a causes HDFN.

Do^a and Do^b of the Dombrock system represent a single amino acid substitution on the Dombrock glycoprotein. Approximately 67% of northern Europeans are Do(a+) and 82% Do(b+). Dombrock antibodies are extremely rare, immune and are best detected by IAT; they have been implicated in severe acute and delayed HTRs. Dombrock typing reagents are rare and unreliable, so typing is best achieved by molecular methods.

Co^a and Co^b of the Colton system represent a single amino acid substitution in the water channel aquaporin-1. Colton phenotype frequencies in white people are as follows: Co(a+b−), 90.5%; Co(a+b+), 9.0%; Co(a−b+), 0.5%. Colton antibodies are very uncommon. Anti-Co^a has caused severe HDFN and has been implicated in acute and delayed HTRs.

Yt^a and Yt^b of the Yt system represent a single amino acid change in red cell membrane acetylcholinesterase. Yt^a and Yt^b have frequencies of about 99.8 and 8% respectively. Anti-Yt^a and anti-Yt^b are exceptional and most are of no clinical significance.

There are other antigens, of either very high or very low prevalence, (classified by the International Society for Blood Transfusion, the ISBT, into the 901 series and 701 series respectively) and those with a serological or biochemical relationship but not yet assigned to blood group systems (collections, termed the 200 series). Some have caused HDFN and/or HTRs. Within other blood group systems, Jr^a, Lan, At^a, MAM and AnWj are clinically significant antigens of very high prevalence. Anti-Vel, an antibody to an antigen of high prevalence causes severe HTRs.

For those rare individuals who have formed antibodies to high-prevalence antigens, the provision of compatible blood can be a problem and it is often necessary to approach the national rare donor registries or, when rare units cannot be sourced nationally, the International Rare Donor Panel for compatible units. Antibodies to low-prevalence antigens are usually naturally occurring; they may occasionally give rise to unexpected incompatible cross-matches. Some antibodies to low-prevalence antigens have caused HDFN.

Polyagglutinable red cells

Erythrocyte polyagglutination is the agglutination of red cells irrespective of blood group by many sera from normal adults. Polyagglutinable red cells are not agglutinated by the patient's own serum. The abnormality is a property of the red cells, not of the sera, in contrast to panagglutination, which is the agglutination of most red cells by one serum.

There are two main categories of polyagglutination, acquired and inherited. The acquired forms can be subdivided into: (i) microbial polyagglutination, which results from the action of microbial enzymes on red cell surface oligosaccharides (T, Tk, acquired B); and (ii) non-microbial polyagglutination, which is caused by somatic mutation (Tn). There are four types of inherited polyagglutination: Cad (strong expression of Sd^a), congenital dyserythropoietic anaemia type II (CDAII or HEMPAS), NOR and Hyde Park. Lectins are required for the identification of the different types of polyagglutination.

The biological significance of blood group antigens

The functions of many red cell membrane proteins bearing blood group antigenic determinants are known, or can be deduced from their structure. Some are membrane transporters, facilitating the transport of biologically important molecules through the lipid bilayer: band 3 glycoprotein, the Diego antigen, provides an anion exchange channel for HCO_3^- and Cl^- ions; the Kidd glycoprotein is a urea transporter in red cells and renal endothelial cells; the Colton and GIL glycoproteins are aquaporin water and glycerol channels; JR and Lan are ATP-binding cassette transporters, which translocate various substrates across biological membranes. RhAG probably functions as a channel for rapid CO_2 transfer in and out of the cell. CO_2 in the red cell is converted to bicarbonate ions by carbonic anhydrase II and then transported rapidly out of the cell to the

plasma by band 3, part of the same macrocomplex as RhAG, where it is carried to the lungs.

The Lutheran, LW and Indian (CD44) glycoproteins are adhesion molecules, possibly serving their functions during erythropoiesis. The Duffy glycoprotein is a chemokine receptor and might function as a 'sink' or scavenger for unwanted chemokines. The Cromer and Knops antigens are markers for decay-accelerating factor and complement receptor 1, respectively, which protect the cells from destruction by autologous complement. Some blood group glycoproteins have enzyme activity: the Yt protein is acetylcholinesterase and the Kell protein is an endopeptidase that synthesises endothelin-3, a vasoconstrictor. The C-terminal domains of the Gerbich glycoproteins, GPC and GPD, and the N-terminal domain of the Diego glycoprotein, band 3, anchor the membrane to its skeleton. The carbohydrate moieties of the membrane glycoproteins and glycolipids, especially those of the most abundant glycoproteins, band 3 and GPA, constitute the glycocalyx, an extracellular coat that protects the cell from mechanical damage and microbial attack.

The difference between red cell antigens that represent the products of alleles (e.g. A and B, K and k, Fy^a and Fy^b) is small, often being just one monosaccharide or one amino acid. The biological importance of these differences is unknown and there is little evidence to suggest that one antigen confers any significant advantage over another. Most blood group systems have a null phenotype in which the whole blood group protein is absent from the red cells or any other cells. These usually result from homozygosity for gene deletions or inactivating mutations within the genes. In most cases, individuals with these null phenotypes are apparently healthy, suggesting that whatever the precise function of the missing structure may be, some other structure must be able to substitute in its absence. However, there are exceptions; rare Rh_{null} individuals, who lack RhD and RhCE proteins, as well as RhAG, have chronic haemolysis, which may be compensated by increased red cell production, but may require splenectomy for stabilisation. Absence of the Kx protein causes weakness of expression of all Kell antigens, as a result of linkage between the two proteins in the membrane, but is also associated with neuroacanthocytosis. Very rare patients lacking the RAPH glycoprotein, CD151, have disruption of basement membranes causing hereditary nephritis, epidermolysis bullosa and neurosensory deafness. A patient lacking all Diego blood group system antigens, which function as an anion transporter, only survived with extreme medical intervention. On the other hand, people with the rare Bombay phenotype lack ABH antigens from all cells and tissues, with no apparent ill effect or red cell abnormality.

Some blood group antigens are exploited by pathological microorganisms as receptors for attaching and entering cells. Consequently, in some cases absence of antigens can be beneficial. The Duffy glycoprotein is used by *Plasmodium vivax* to penetrate red cells. Hence, the Fy(a−b−) phenotype, common in Africans, confers resistance to *P. vivax* malaria. *Plasmodium falciparum* malaria appears to have played a part in the establishment of the ABO polymorphism. The geographic distribution of group O is consistent with selection pressure by *P. falciparum* in favour of group O individuals in malaria endemic regions.

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CHAPTER 19

Clinical blood transfusion

19

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Introduction

This chapter covers key aspects of blood donation and collection and the preparation and storage of blood components; it describes pretransfusion testing of donors' and recipients' blood, complications and adverse effects of blood transfusion, as well as the appropriate use of components and transfusion alternatives available in different clinical scenarios. The features of haemolytic disease of the foetus and newborn are also described. The regulatory and logistical descriptions mainly reflect a UK perspective, although some international considerations are made, and the principles for clinical practice are, to a great degree, universal.

Blood transfusion and regulatory aspects

Concerns about blood safety and in particular transfusion-transmitted infection have resulted in an increasingly stringent regulatory framework for transfusion medicine. The World Health Organisation (WHO) and the Council of Europe (CoE) have defined a code of ethical principles which serve as guidance for countries on blood donation and transfusion practice. The European Union (EU) Blood Directives set standards of quality and safety for the collection, testing, processing, storage and distribution of blood and components. The Good Practice Guidelines, prepared through co-operation between the European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM) and the Commission of the European Union (EU), form an integral part of the 20th edition of the COE Guide to the preparation, use and quality assurance of blood components. In the UK these EU Directives have been transposed into law as the Blood Safety and Quality Regulations 2005. Aspects of the regulations apply to 'blood establishments' (the UK Blood Services) and hospital blood banks, and the Acting Competent Authority under these regulations is the Medicines and Healthcare products Regulatory Agency (MHRA) which regulates the Blood Services and hospital transfusion laboratories in the UK.

The blood donor

An adequate and reliable supply of safe blood can be assured by a stable base of regular, voluntary, unpaid blood donors who are also the safest group of donors with the lowest prevalence of blood-borne infections. World Health Assembly resolution WHA63.12 urges all Member States to develop national blood systems based on voluntary unpaid donations and to work towards the goal of self-sufficiency. However, whole blood donation and apheresis plasma donation are still remunerated in some countries.

Selection criteria and blood donation

Donor selection criteria are aimed at ensuring the safety of the donor and minimising risk to recipients, in particular the risk of the transmission of infection. Blood donors should be healthy adults within a permitted age range with minimum requirements for weight, haemoglobin, donation volume and donation frequency (see Table 19.1).

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 19.1 Measures to protect the donor from adverse effects of large-volume blood withdrawal.

- Age 17–70 years (60 at first donation).
- Weight above 50 kg
- Haemoglobin >135 g/L for men, 125 g/L for women for whole blood and all component donors except those who only donate plasma by apheresis
- Minimum donation interval of 12 weeks (16 weeks for females)
- Pregnant and lactating women excluded because of high iron requirements
- Exclusion of those with:
  - Known cardiovascular disease e.g., ischaemic heart disease
  - Significant respiratory disorders
  - Epilepsy and other CNS disorders
  - Inflammatory bowel disease
  - Insulin-dependent diabetes
  - Chronic renal disease (CKD ≥ 3)
  - Ongoing medical investigation or clinical trials
- Exclusion of any donor returning to occupations such as driving bus, plane or train, heavy machine or crane operator, mining, scaffolding, etc. (delayed faint risks)

The minimum age for blood donation in many countries is 17 years, essentially aimed at avoiding donation in adolescence when iron requirements are high. Similarly, pregnant and lactating women are not permitted to donate. In the UK, the upper age limit for first-time donors is 65 years, with no upper age limit for regular donors in good health. Screening of blood donors should be carried out by a trained assessor, to ensure that the donation will not compromise the health of the donor, the health of the recipient or the quality of the blood collected. A full donor medical history will help identify those not suitable for donation (e.g. those with cancer, cardiovascular or renal disease). A full travel history including long stay/residency is essential together with any activities known to be associated with increased risk of acquiring infections with temporary or permanent deferral as needed (see below). Due to the risk of delayed faints, donors in hazardous occupations or hobbies (e.g. scaffolding, firemen, climbers) should not be allowed to return to work or their hobbies until the following day.

Donor haemoglobin screening is an important part of donor assessment. The haemoglobin level is assessed prior to each donation usually by a semi-quantitative, gravimetric method using a drop of capillary blood in a copper sulfate solution, but this may be supplemented by use of portable haemoglobinometers. The minimum acceptable haemoglobin level varies: it is 125 g/L for females and 135 g/L in males for most donations including whole blood and all component donors (except those who only donate plasma by apheresis, in whom a lower Hb may be tolerated). No more than 15% of the estimated blood volume should be taken during any one donation and, in general, 450 mL+/–10% of blood is collected with an interval of 12–16 weeks between whole blood donations. Individual components such as platelets and plasma can also be collected by apheresis using a cell separator with a maximum of 24 procedures in 12 months.

The appropriate frequency of blood donations has been investigated by the INTERVAL study which examined donation at 8-, 10- and 12-week intervals for men, and 12-, 14- or 16-week intervals for women. Although giving blood at the shorter intervals resulted in much more blood being collected without a major impact on the donors' quality of life, mental function or physical activity, some of those who gave blood more frequently did report minor symptoms including tiredness and restless legs. Haemoglobin estimation using the copper sulfate method failed to identify up to 10% of people with haemoglobin levels below the required limit, putting donors at risk of developing anaemia and iron deficiency. Portable haemoglobinometry (i.e. using capillary HemoCue) has been shown to have the highest accuracy across all haemoglobin thresholds examined for both men and women, as well as the smallest biases in comparison with the reference standard.

Donors with minor red cell abnormalities, such as thalassaemia trait and hereditary spherocytosis, are acceptable, providing the haemoglobin screening test excludes anaemia and donors have themselves not needed any transfusion support. Red cells from donors with sickle cell trait containing HbS may block leucodepletion filters and have limited survival under conditions of reduced oxygen tension and so should not be transfused to newborn infants and patients with sickle cell disease. Red cells from donors with glucose-6-phosphate dehydrogenase deficiency survive normally, unless the recipient is given oxidant drugs. Individuals with hereditary haemochromatosis are accepted for donation provided they meet other donor selection criteria and are under ongoing medical review.

Donor selection guidelines are reviewed regularly and, based on new evidence as well as estimated risks, these are updated regularly. In November 2017, the blood donor selection guidelines for men who have sex with men (MSM) were changed in England, Wales and Scotland from a 12-month deferral since last sex with a man to a 3-month deferral. Similar changes were made for other donor selection criteria related to higher-risk sexual behaviours. The For the Assessment of Individualised Risk (FAIR) steering group recommended a more individualised approach to donor selection in the UK. Under this new donor selection policy, donors who have had the same sexual partner (and no others) in the last 3 months and who do not have a sexually transmitted infection should be eligible to donate, allowing more gay, bisexual and men who have sex with men to donate blood.

Donated blood is not tested routinely for the SARS-CoV-2 virus that causes COVID-19 and, to date, there are no known cases of transfusion-transmitted SARS-CoV-2 infections reported and there is currently no evidence that SARS-CoV-2 is a transfusion-transmitted infection.

Transfusion-transmitted infection (TTI)

Several infections have the potential to be transmitted by transfusion of blood or its components (see Table 19.2). The epidemiology of infection in the population of a particular country can help guide the microbiological testing required. Careful donor selection criteria, with detailed questionnaires, aimed at excluding individuals at high risk of carrying infection, are supported by stringent microbiological testing (Table 19.3) with additional use of measures where appropriate to protect the recipient (see Table 19.4). Despite these measures, infection may still be transmitted, although rarely, due to the donor being in the incubation or 'window period' before testing becomes positive for infective markers or due to transmission of infections of unknown aetiology, or where no screening test is available to date (e.g. prion disease).

The viruses that pose the greatest potential risk for transmission by transfusion are those that have long incubation periods (often causing subclinical infection), and especially those that may be carried by asymptomatic individuals for many years, or even lifelong. Alternatively, some viruses that are transfusion-transmissible exhibit cell-associated latency. If the virus is latent in white blood cells, recrudescence of that virus, stimulated by allogeneic transfusion, can cause infection of the recipient. More rarely, a few viruses causing acute infection can be transmitted in the short presymptomatic-viraemic phase, if this coincides with the time of blood donation.

Some groups of people may be at particular risk of blood-borne infections, people who inject drugs and individuals paid to have sex, who are accordingly asked never to give blood. Temporary deferrals may also be applied in certain circumstances for example; in the UK, potential blood donors cannot donate for at least 3 months if they have taken part in chemsex activity, including the use of stimulant drugs.

The chance of not detecting and releasing an infectious donation made by a donor who has very recently acquired hepatitis B, hepatitis C or HIV is estimated over a rolling 3-year period each year. The current estimate of a window period donation being not detected on screening remains at less than one in a million, with the risk being highest for Hepatitis B. There is

Table 19.2 Transfusion-transmissible agents.

Agents		Characteristics related to transfusion
Viruses		
Hepatotropic	HAV	Very rarely transfusion transmitted (rare in the UK, generally imported cases) during incubation; no carrier state; faecal-oral transmission
	HEV	As above but person-to-person spread is rare
	HBV	2–6-month incubation period; carrier state; readily transmissible by blood
	HCV	Majority of cases asymptomatic; carrier state; readily transmissible by blood
Retroviruses	HIV-1 and HIV-2	Carrier state and latent in WBCs; readily transmissible by blood
	HTLV-I and HTLV-II	Latent in WBCs
Herpesviruses	CMV	50% of UK adults have been infected; latent in WBCs
	EBV	Most UK adults have been infected (therefore already exposed pretransfusion); latent in WBCs
Others	Parvovirus B19	Generally mild or asymptomatic, posing no transfusion risk except for non-immune aplastic anaemia patients, haemoglobinopathies and foetuses Approximately two-thirds of UK adults have been infected Seasonal variation (and epidemic years) in incidence rate
	West Nile virus	Recently exhibiting epidemic rates of transmission in summer months in North America
	Dengue virus	Transfusion transmission has been reported in Asia
Bacteria	*Treponema pallidum*	Inactivated by storage at 4 °C
Endogenous		
	Yersinia enterocolitica	Very occasional transmissions, usually contaminated red cells transfused late in the storage period
	E. coli	Very occasional transmissions, usually contaminated red cells transfused late in the storage period
Exogenous	e.g. *Staphylococcus epidermidis*, *Micrococcus*, *Sarcina*	Mainly skin commensals or contaminants Most common cause of platelet contamination
Parasites	Malaria	Frequently transmitted in endemic countries in Africa, Asia and the Caribbean.
	Chagas disease	No transmission of *Trypanosoma cruzi* by transfusion has been reported in UK; many cases in Latin America
Prions	Abnormal PrP	Transfusion risk from vCJD.

WBCs, white blood cells.

Table 19.3 Microbial testing in England.

HEV	RNA screening in pools
HIV	ELISA (combined HIV-1 antigen plus anti-HIV-1 and anti-HIV-2); NAT for RNA
HBV	HBsAg ELISA; NAT for DNA; anti-HBc on new donors
HCV	Anti-HCV ELISA plus NAT for RNA
HTLV	Anti-HTLV ELISA (on pools of samples)
CMV	Anti-CMV for selected immunosuppressed recipients only
Malaria	Antibody screening of potentially exposed donors
Chagas disease	Antibody screening of potentially exposed donors
Bacteria	All donations are tested for antibody to syphilis; all platelet preparations are tested by culture methods

Table 19.4 Measures to protect the recipient.

Measure	UK currently
Donor selection	Yes
Donor deferral/exclusion	Yes
Stringent arm cleansing	Yes
Diversion of the first 20–30 mL of blood collected	Yes
Microbiological testing of donations	Yes
Immunohaematological testing of donations	Yes
Leucodepletion of cellular blood components	Yes
Safest possible sources of donors for plasma-based products	For at-risk patients
Monitoring and testing for bacterial contamination	Yes
Pathogen inactivation of cellular components (e.g. psoralens)	No
Prion filtration	No

routine surveillance of all reported suspected TTIs to the blood services for full investigation.

Hepatitis viruses

Hepatitis viruses can be transmitted in cellular and plasma components, as well as plasma products, and currently across UK it's a permanent deferral post introduction of anti-HBc, other countries will allow donation with sufficient immunity. Since for hepatitis A, there is no carrier state and any transmission can be possible only during the incubation or acute phase, deferral is also required for a donor who has been in close contact with a case (e.g. household contact) as well as those who have developed hepatitis A within the last 12 months. HEV and HAV have a 6-month deferral post recovery now in the UK.

For hepatitis B, C and E which have carrier/chronic states, further testing is required. With hepatitis E, the chronic state is usually only in immunosuppressed individuals. For hepatitis B, all donations are tested for the presence of HBsAg by sensitive ELISAs that can detect at least 0.2 IU/mL of HBsAg. More sensitive screening tests, such as HBV nucleic acid testing (NAT), have been introduced in many countries: this shortens the 'window period' of infectivity. NAT testing is also used for hepatitis C, and the risk of transmission by blood transfusion has fallen dramatically since its introduction.

Hepatitis B, historically acute infections, most recently occult hepatitis B infections, are remains the most reported viral TTI in the UK because of window-period transmissions either during the incubation period or at the tail end of the carrier state. More recently, the specialist advisory committee on the safety of blood, tissues and organs (SaBTO) has recommended that all donors be tested at least once for anti-HBc, a marker of hepatitis B infection. Individuals who have recovered from hepatitis B will remain anti-HBc positive. There is a risk that reactivation of hepatitis B virus in such an individual could give rise to occult hepatitis B infection which is not detected by routine testing. For this reason, donors who are anti-HBc positive are deferred from donation. UK Blood Transfusion Services are currently implementing this.

For Hepatitis E: there have been several reported cases of transfusion-transmitted HEV following transfusion of red cells, platelet preparations, pooled granulocytes and fresh-frozen plasma. Because of the rising trend of HEV serological prevalence amongst the global population, and the fact that transfusion-transmitted-HEV infection can cause serious clinical consequences amongst those patients most in need of blood transfusion, universal hepatitis E screening was introduced in the UK in 2017. The screening approach, using pools of 24 donations, that is currently in operation to detect hepatitis E viraemia in donated blood is likely to detect the majority of viraemic donors. Those with viral loads below the level of detection remain a potential risk, though the likelihood of transmission from components containing very low levels of HEV RNA appears small. A total of 12 incidents (15 recipients) have been reported relating to HEV transmission through transfusions in the UK resulting in 2 deaths and 9 cases with major morbidity related to the viral infection. All except 2 HEV transmissions in the UK were reported before HEV RNA screening was introduced in April 2017 infections tend to be with genotype 3 in the UK.

Human immunodeficiency virus (HIV-l and HIV-2)

HIV can be transmitted in both cellular and plasma components, and blood services have robust processes to minimise this risk. Most patients infected by the transfusion of blood components were transfused before the introduction of screening of blood donations for HIV antibodies. Moreover, most recipients

of fractionated plasma products infected in the past were transfused before 1985 with unheated, non-Pasteurised pooled plasma products, mainly factor VIII and factor IX concentrates. Thus, HIV infection became an important sequel to transfusion of factor VIII concentrates to people with haemophilia in the early 1980s. HIV is more heat labile than HBV, especially when in solution and is also susceptible to other viral inactivation methods such as solvent-detergent treatment.

Every donation should be tested for antibodies to HIV-1 and HIV-2 and common subtypes, using a test system such as ELISA. Laboratory markers for HIV include anti-HIV, p24 antigen and viral RNA. To reduce the window period for HIV, many blood centres use combination tests for HIV antibody and p24 antigen, and/or perform NAT testing in addition to antibody testing.

Every donation in the UK is tested for antibodies to HIV (anti-HIV), and combined antigen–antibody for HIV (HIV Ag/Ab). Donated blood in the UK is tested in pools of 24 samples by multiplex HBV, HCV and HIV NAT as well. Routine screening, combined with well-established donor education and self-deferral schemes, can reduce the already small risk of HIV transmission by transfusion in those countries where blood donors are voluntary and mostly regular.

Human T-cell leukaemia viruses

Human T-cell leukaemia viruses (HTLV-I and HTLV-II) are related retroviruses. Infection with HTLV-I is associated with at least two distinct clinical conditions: it can occasionally lead to adult T-cell leukaemia, with an incubation period of approximately 20 years, and, on even rarer occasions, to tropical spastic paraparesis (also known as HTLV-I-associated myelopathy), with a shorter incubation period. Only about 1% of patients who are seropositive develop T-cell leukaemia.

Both HTLV-I and HTLV-II are cell-associated and not transmitted in plasma. In highly endemic areas, transmission of HTLV-I by transfusion was relatively common before mandatory screening was introduced. Both tropical spastic paraparesis and adult T-cell leukaemia have been associated with transfusion-transmitted HTLV-I. In England and Wales, an estimated 22,000 people are living with the virus, mainly those who have migrated from endemic countries, or their descendants. In the UK, screening using a sensitive ELISA for anti-HTLV-I/II on pooled plasma samples became mandatory in 2002. Additionally, universal pre-storage leucoreduction results in a significant reduction in the rate of transmission. If a donation tests positive for HTLV-I, -II it must be discarded, the donor notified and informed and referred to specialist care, and asked never to donate again.

Cytomegalovirus

Although most cases of post-transfusion cytomegalovirus (CMV) infection are subclinical, the syndrome of post-transfusion infectious mononucleosis-like illness is well recognised, especially after the transfusion of large amounts of blood. The infection is characterised by fever, splenomegaly and atypical lymphoid cells in the peripheral blood, with a negative Paul Bunnell test. The usually benign course of CMV infection in most recipients and the high prevalence of CMV antibodies in the general population have meant that there has been no necessity to screen all donors for evidence of past infection. Immunosuppressed individuals are at greatest risk from potentially fatal pneumonitis or disseminated CMV infection.

In 2011, following a review of available evidence, SaBTO recommended that leucodepletion of all blood components (other than granulocytes) provided adequate CMV risk reduction for most patients requiring transfusion (haemopoietic stem cell transplant recipients, organ transplant patients and immune deficient patients, including those with HIV) without the requirement to provide them, in addition, with CMV seronegative cellular blood components. CMV seronegative red cell and platelet components should continue to be provided for intrauterine transfusions and for neonates, as well as for pregnant women requiring repeat elective transfusions during pregnancy. Granulocyte components should be provided as CMV-negative for all CMV-negative allogeneic transplant patients (or potential patients) as these components cannot be leucodepleted. A medical decision may be made to transfuse units which are not CMV tested, or which are known to be CMV IgG positive, into a CMV-negative patient if the urgency to treat a non-responsive bacterial or fungal infection outweighs the risks of potentially developing CMV infection at a later stage.

CMV-negative blood components are those collected from donors who have been tested and found negative for CMV IgG antibodies.

Syphilis

According to WHO data, the global incidence of syphilis has increased in recent years. Each donation is tested by a serological test for syphilis. The syphilis spirochete (*Treponema pallidum*) is susceptible to time and temperature and will lose its ability to infect a recipient after several days of storage after donation, particularly if the storage is at 4 ± 2 °C. Serological tests for syphilis include a rapid plasma reagin test (RPR), treponema pallidum haemagglutination test (TPHA) and fluorescent treponemal antibody absorption test (FTA). Any donation from an individual giving a positive result is discarded, and subjects with positive tests are permanently withdrawn from donation, even after effective therapy.

Malaria

Plasmodia remain viable in red cells stored at 4 °C and are readily transmissible by blood transfusion. Red cells and whole blood are most likely to transmit infection but this is possible

with all cellular blood components. Transfusion-transmitted *Plasmodium falciparum* may cause severe malaria. Maintaining a malaria-free blood supply is an ongoing challenge for many blood services as malaria in asymptomatic carriers is difficult to detect due to the very low level of parasitaemia and blood donors from endemic areas often have circulating antibodies as a result of partial immunity to malaria.

In some endemic areas, all recipients are treated with antimalarial drugs. In non-endemic areas, there is a real risk of failure to recognise post-transfusion malaria owing to the rarity and unexpectedness of the infection. This fact, combined with increasing travel to tropical areas, necessitates the careful vetting of blood donors by direct questioning and, in some centres, by tests for malarial antibodies. In the UK, donors with a history of possible malaria exposure (provided it was more than 4 months previously and they are free of symptoms) can be accepted if a validated test for malarial antibody is negative. Donors who have had malaria diagnosed in the past can be accepted for blood donation if more than 3 years have passed since antimalarial therapy has been completed, symptoms caused by malaria have resolved and a validated test for malarial antibody is negative. Pathogen reduction methods that can be performed on whole blood or red cells are under development and show promise in reducing transmission.

Other infections

Acute viral infections, although associated with short phases of viraemia, can pose transmission risk by transfusion especially if their incidence is high. This has been demonstrated by West Nile virus (WNV) in North America. WNV is an arthropod-borne infection of birds, with humans and horses as incidental hosts. The annual outbreaks in recent years in the USA and Canada have resulted in several transmissions by transfusion, with severe morbidity and mortality in immunosuppressed and elderly patients. The EU Blood Safety Directive (and the Blood Safety and Quality Regulations) requires that travellers from an area with ongoing transmission of WNV in humans should be deferred for 28 days. Outbreaks of WNV infection in a number of areas within Europe in 2010/11 led to the introduction of WNV NAT testing of donations in affected areas.

Arboviruses, such as dengue, though rarely, can be transmitted by transfusion. Chikungunya virus might be transmissible.

Diseases such as Chagas disease cause significant problems for the blood transfusion services in Latin America and, due to immigration, in other areas. Potential exposure of UK donors necessitates stringent donor selection criteria and specific serological testing of those individuals to exclude the possibility of infection. *Babesia microti*, the agent of Nantucket fever, still poses a risk in certain areas of the USA. There are very few reports of possible transmission of *Leishmania donovani*, all outside Europe. Subjects giving a history of brucellosis are declined as donors in the UK due to the risk of transmission of this agent.

Bacteria

Bacteria from the donor arm can contaminate blood components. Rarely, low-grade or asymptomatic infections or translocated organisms from the gut in the donor, with *Salmonella* or *Yersinia*, may be an endogenous source of contamination of blood components. Coagulase-negative staphylococci from normal skin flora are rarely pathogenic, whereas *Staphylococcus aureus*, and Gram-negative organisms, such as *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp., may result in life-threatening infections. Platelet components are at particular risk due to their storage at 20–24 °C, the risk increasing with length of storage. It is estimated that up to 1 in 2000 platelet packs contain detectable bacteria 5 days after donation and in the past, fatal reactions have been reported in 1 in 25,000–80,000 transfusions. Most pathogenic bacteria grow poorly in refrigerated temperatures needed for red cell storage, but some Gram-negative organisms, such as *Yersinia enterocolitica* and *Pseudomonas* spp., can grow under these conditions.

Bacterial contamination of blood has been significantly reduced in the UK by the introduction of improved donor arm cleansing using 70% isopropyl alcohol/2% chlorhexidine gluconate applied as a single-step procedure, and diversion of the first 20–30 mL of the blood donation. The risk of bacterial contamination has been further reduced, but not eliminated, by routine bacterial screening of platelet preparations with extension of shelf life from 5 to 7 days. The four UK Blood Services all use the BacT/ALERT system for bacterial screening which has been successful in reducing the risk of bacterial TTI (McDonald et al. 2017).

It is important to recognise that screening of platelet components cannot guarantee the absence of bacterial contamination. Packs are released for issue as 'negative-to-date', which may be before bacteria have multiplied sufficiently to trigger detection on screening. There have been ten bacterial near misses, all but one in platelet components, reported between 2011 and 2020 in the UK. Overall, out of a total of 44 bacterial transfusion transmissions to individual recipients, 37 (34 donations) have been caused by the transfusion of platelets, and 7 by red cells since reporting began in 1996.

Pathogen inactivation (PI) technologies for platelets and red cells, such as the use of light-activated psoralens that kill organisms by damaging their DNA or RNA have the potential to eliminate both bacterial and viral transfusion-transmitted infections. Whilst PI methods are now available, mostly for platelets, the cost-effectiveness of these techniques, together with the long-term risks, are uncertain, with the loss of viable platelets being a concern. Accordingly, although some parts of Europe have introduced PI, the potential for wide implementation in the UK is still under review.

Prions

Variant Creutzfeldt–Jakob disease (vCJD), the human form of bovine spongiform encephalopathy (BSE), was considered a potential threat to blood safety, particularly applicable to the UK.

To date, since 1996, when vCJD was first reported, there have been only four cases in the UK where blood transfusion of non-leucodepleted red cells may have been implicated in transmission of vCJD. All four cases occurred before 2007. A further possible transmission of vCJD prions was reported in February 2009 in a patient with haemophilia who had received batches of factor VIII to which a donor who subsequently developed vCJD had contributed plasma. The patient died of other causes but was found to have evidence of abnormal prion accumulation in his spleen.

There is no blood test at present for detecting prions in blood donations. The full risk of vCJD in the UK population is now thought to be low but the UK blood services have taken a number of precautionary measures to reduce the potential risk of transmission of prions by blood, plasma and blood products. These include: (i) barring various individuals from blood donation, such as those who have received a blood transfusion or tissue/organ transplant since 1980 or anyone who has received human pituitary-derived hormones or grafts of human dura or ocular tissue, and (ii) universal leucocyte depletion of all blood donations since 1999. Two other risk-reduction measures have now been withdrawn due to the re-assessed low risk of vCJD transmission: discarding UK plasma and importing plasma from countries other than the UK for fractionation into plasma products (withdrawn 2021), and importation of fresh-frozen plasma for use in patients born after January 1996 (withdrawn 2019).

Laboratory tests on blood donations

Samples for laboratory testing are taken at the time of donation in order to avoid later entry into the sterile blood pack. The routine tests are automated if large numbers of donor samples are tested daily, as is the case in the UK.

All blood donors in the UK are tested at each donation for syphilis, HBsAg, anti-HIV-1 and anti-HIV-2 and HIV antigen, anti-HCV, HCV and HIV RNA, HBV DNA and anti-HTLV. Anti-HTLV screening is only required for blood donations from previously untested donors and for blood donations destined for use to prepare non-leucodepleted products. Some donations are screened for CMV antibodies for patients in need of CMV-negative blood (as discussed previously). Additional testing may be needed of donors at particular risk based on travel history e.g. malaria antibodies, *T. cruzi* (Chagas) antibodies.

ABO and RhD grouping are determined routinely on each occasion. Typing for other Rh antigens (C, E, c and e) and K is now routinely performed on all blood donations in the UK. Phenotyping for other red cell antigens such as Duffy, Kidd and MNSs is performed on a restricted number of units in order to provide antigen-negative blood for alloimmunised patients and for transfusion-dependent patients likely to make multiple antibodies, such as those with sickle cell disease. Ideally, girls and women of childbearing age should be matched for c and K, as anti-c and anti-K are, after anti-D, the major causes of severe haemolytic disease of the newborn (HDN). In the UK, it is now recommended practice to give K-negative blood to females of childbearing potential to minimise the risk of forming anti-K.

All donations are also screened for the presence of atypical red cell alloantibodies. The incidence of clinically significant red cell alloantibodies in blood donors is very low (<0.3%) compared with the incidence in potential recipients (1–2%). Donations with potent clinically significant alloantibodies are not issued to hospitals. There are additional requirements for blood provision for neonatal and intrauterine use, including the use of repeat, rather than new, donors, HbS-negative and exclusion of high-titre anti-A and -B. Group O blood for emergency or 'flying squad' use, which may be given in an emergency to ungrouped recipients, should be given as red cells in additive solution such as saline-adenine-glucose-mannitol (SAGM) in order to avoid problems that might be caused by high-titre anti-A, B in donor plasma. As soon as the patient's group is known, group-specific blood should be given. If group O platelets have to be given to non-O recipients, donors with high-titre anti-A and B should be excluded as the providers of the plasma used to suspend the platelets; alternatively, platelet-additive solutions can be used to replace most of the plasma.

Molecular techniques are becoming increasingly available for blood group testing and extended red cell genotyping; they are likely to come into more routine use within blood services with the application of rapid automated technology.

Storage and processing of blood

Donor blood is collected into plastic packs containing anticoagulants and then transported without delay to the blood centre for processing. In the UK, all units undergo leucodepletion at different stages of processing, depending on the final components required (see below). Processing (see Figures 19.1 and 19.2) is undertaken to produce red cells, platelets and plasma or cryoprecipitate under stringent standards of quality control, as mandated by the Blood Safety and Quality Regulations 2005. Anticoagulants and preservatives prevent clotting and maintain cell viability and function during component storage. Storage conditions relate largely to the maintenance of temperature from the time of collection, through processing, testing and labelling and transportation, up to the point of issue for transfusion into a patient. This is known as 'cold chain management'.

Storage changes

Loss of red cell and platelet viability are the most important practical considerations. Progressive loss of viability varies according to the combination of anticoagulant used, how blood is stored prior to component separation, what storage medium the components are stored in, and the pack systems used. The time limit for storage of blood or its component parts is set,

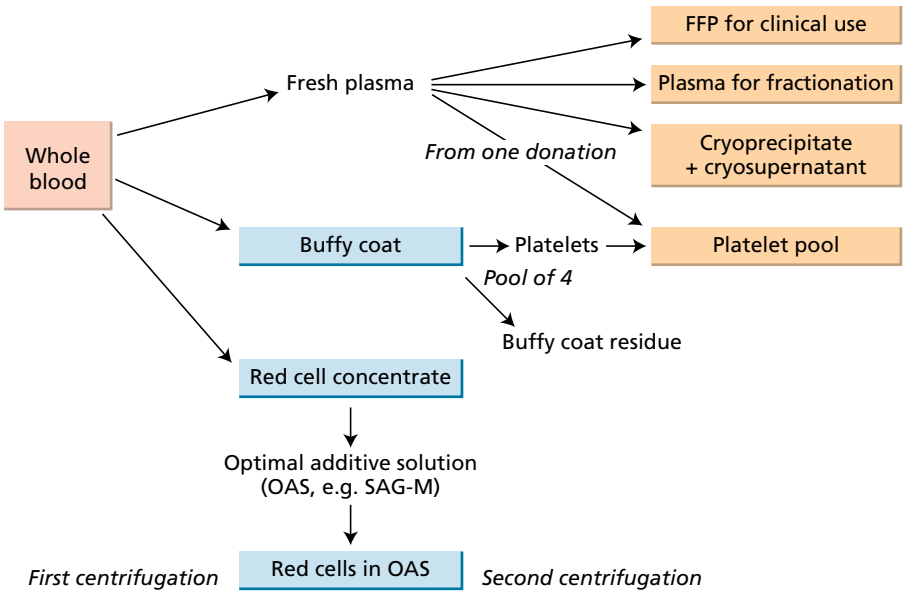

Figure 19.1 Diagrammatic representation of the preparation of components from whole blood by the 'top and bottom' or 'buffy coat' method. Items in boxes represent final components. One unit of plasma is no longer used for platelet pooling, instead a pack of Platelet Additive Solution (PAS) is used.

taking all these into consideration. After transfusion of stored red cells or platelets, a proportion is removed from the circulation within the first 24 hours. The remainder appear to survive normally. There are various changes in the *in vitro* characteristics of red cells during storage (the so-called storage lesion), including depletion of metabolic substrates such as adenosine triphosphate (ATP) and 2,3-diphosphoglyerate (2,3-DPG), leakage of potassium and changes in red cell morphology. Some of these changes can be partially reversed *ex vivo* by incubation with purine nucleosides, or *in vivo*, following transfusion. ATP seems to be an important determinant of red cell viability, although not the only one. 2,3-DPG is almost completely

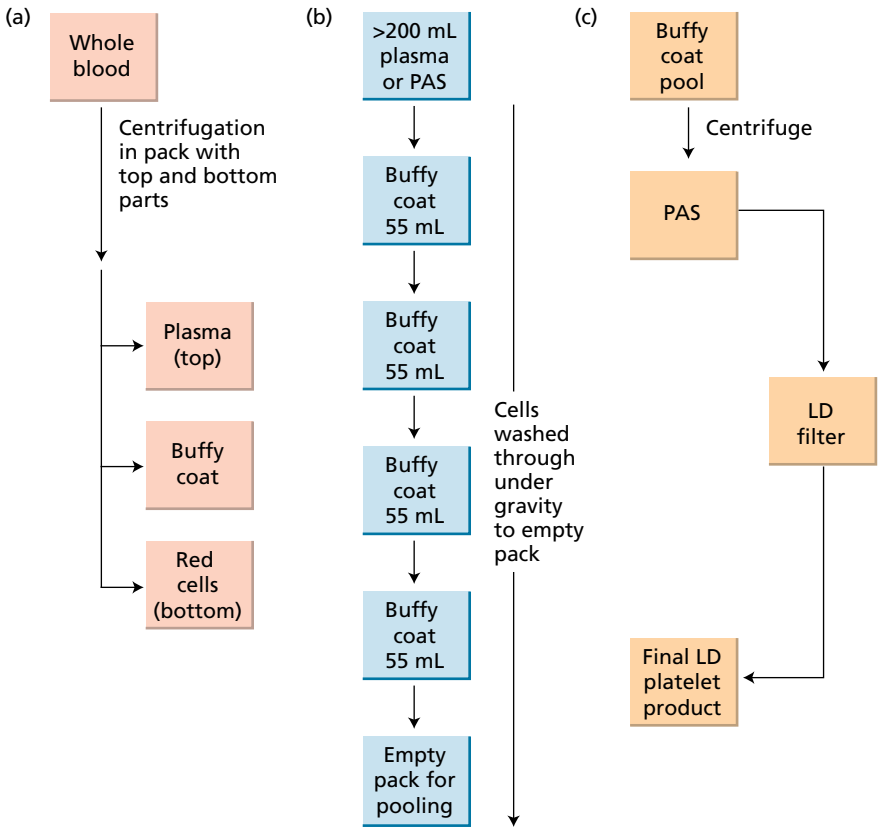

Figure 19.2 The 3 stages, a-c, involved in the manufacture of leucodepleted pooled, buffy-coat-derived platelet preparations. After gentle centrifugation of the buffy coat pool, the platelets remain in the plasma supernatant and the buffy coat residue is discarded. LD, leucodepletion; PAS, platelet-additive solution.

depleted in red cells after 14–21 days of storage. Red cell 2,3-DPG is restored to normal by approximately 24–48 hours after transfusion. The clinical significance of the low 2,3-DPG level of stored red cells is unknown, but only likely to be an important consideration in recipients with severe anaemia or coronary artery insufficiency.

During storage of red cells, potassium gradually leaks out through the cell membrane once active transport has been halted by the cooling of blood to 4 °C. There is rapid restoration of electrolyte levels after transfusion. The pH of blood decreases with storage, but most recipients can handle the acid load during transfusion without ill effect. Likewise, a number of changes in platelet function occur during platelet storage, including increased platelet activation and decreased responsiveness to agonists such as ADP. For both red cells and platelets, the relationship between these changes *in vitro* and the function of the cells following transfusion is complex, and it is notoriously difficult to predict post-transfusion viability from the results of laboratory testing.

Anticoagulants and optimal additive solutions

When blood is stored in a liquid state, there is a progressive loss of viability of the red cells, red cell ATP and depletion of 2,3-DPG. The purpose of modern anticoagulants used for the collection of blood, and additive solutions to store red cells and platelets, is to reduce these changes to a minimum.

Anticoagulant and preservative solutions vary depending on their composition. All anticoagulant preservative solutions contain sodium citrate, dextrose and citric acid, and some may, in addition, contain phosphate and adenine. The volume of anticoagulant required to prevent clotting and preserve red cells is dependent on the volume of blood collected from the donor. Most whole blood donations are collected into citrate-phosphate-dextrose (CPD) anticoagulant. Platelets from apheresis are collected into acid-citrate dextrose. Optimal additive solutions such as SAG-M containing saline, adenine, glucose and mannitol have been developed to improve the viability of plasma-depleted red cells on storage, and this is now the usual preservative solution for red cells in the UK. Due to theoretical concerns of adenine and mannitol toxicity, CPD continues to be used for the relatively large volume of red cells used for neonatal exchange transfusion and for intrauterine transfusion (no SAGM is added and the red cells remain suspended in plasma), with SAG-M red cells used for top-up neonatal transfusions.

Leucodepletion

Many developed countries have introduced universal leucocyte depletion of blood components. In the UK, leucodepletion of all allogeneic blood components, to a level below 5×10^6 white cells per unit, has been mandatory since 1999, as a measure which might decrease the risk of vCJD transmission by transfusion. Added benefits of leucodepletion include a significant decrease in the incidence of febrile non-haemolytic transfusion reactions (FNHTR), in the risk of human leucocyte antigen (HLA) alloimmunisation and in the risk of transmission of intracellular viruses such as CMV and HTLV. The UK specification for leucodepletion is that more than 90% of leucocyte-depleted components from relevant processes should have less than 1×10^6 leucocytes and more than 99% of components should contain less than 5×10^6 leucocytes, both with 95% confidence. Process performance should be assessed against the 1×10^6 limit when using statistical process control (statistical process monitoring) measurements.

Leucocyte depletion can be achieved by several methods, which must be validated before use. If filtration is used the recommended capacity of the filter must not be exceeded. Leucodepletion filters can be used at the initial stage on whole blood, leaving leucodepleted red cells and plasma, or later in the processing, for the preparation of platelet concentrates from pooled buffy coats, when the final components, red cells, platelets and plasma must be filtered individually (see Figures 19.1 and 19.2). These two alternative methods are necessary because whole blood filters remove a significant number of platelets, limiting production of platelet concentrates from filtered whole blood. Leucocyte depletion of components should take place before the end of day 2 (day 0 is the day of collection).

Blood components

Whole blood

At present, there is no whole blood (WB) product manufactured by the UK Blood Services, although there is increasing interest in the use of WB in a civilian pre-hospital setting, trail soon to be completed. Within international military settings, it has remained an option, and some civilian blood services across the world have introduced its use, particularly in health care settings with dispersed and remote populations.

Red cells

The vast majority of donor blood is separated into its components. A multiple 'bottom and top' blood collection pack is used (Figure 19.1) if the donation will be used to make platelets; otherwise, a simpler 'top and top' system is used to make red cells and plasma. In the bottom and top system, after centrifugation, whole blood collected in CPD is separated into plasma at the top, a buffy coat in the middle and red cells at the bottom (Figure 19.1). Fresh plasma is expressed from the top and red cells from the bottom of the pack, leaving the buffy coat in the original pack. The red cells are transferred to a pack containing an optimal additive solution to preserve red cell function during storage and are also filtered. The resulting red cells have a shelf

life of 35–42 days (35 days in the UK). Adult red cells in SAG-M in the UK have a volume of 220–340 mL with haematocrit of 0.5–0.7, minimum Hb of 40 g/unit and a residual plasma volume of 5–30 mL.

The specification for red cells for intrauterine (IUT), neonatal and infant transfusion is shown in Table 19.5.

Table 19.5 Specifications of red cells for foetal/neonatal/infant transfusion.

Red cell components for IUT
Plasma reduced (haematocrit 0.7–0.85)
In citrate phosphate dextrose (CPD) anticoagulant (theoretical risk of toxicity from additive solutions)
Leucocyte-depleted
Less than 5 days old (to avoid hyperkalaemia)
Cytomegalovirus (CMV)-antibody negative
Sickle screen negative
Irradiated to prevent TA-GvHD (shelf life 24 hours)
Usually group O with low-titre haemolysins
RhD- and K-negative and red cell antigen-negative for maternal alloantibodies
Compatible with the mother's plasma by an IAT cross-match
Red cells for neonatal exchange transfusion
Plasma reduced with haematocrit of 0.5–0.6 to reduce the risk of post-exchange polycythaemia
In CPD anticoagulant
Less than 5 days old
Irradiated (essential if previous IUT)
CMV-antibody negative
Sickle screen negative
Usually produced as group O (with low-titre haemolysins)
RhD negative (or RhD identical with neonate) and Kell negative
Red cell antigen-negative for maternal alloantibodies
Compatible with the maternal plasma by IAT cross-match
Red cells for small-volume transfusion of neonates and infants ('Paedipacks' – 6 splits from single unit)
Haematocrit 0.5–0.7
In SAG-M anticoagulant/additive solution (approximately 20 mL residual plasma)
Up to 35 days from donation
Group O (or ABO-compatible with baby and mother) and RhD negative (or RhD compatible with the neonate)
CMV-negative
If irradiated, 14-day shelf life
Red cells for large-volume transfusion of neonates and infants ('LVT') eg cardiac surgery
Less than 5 days old
If irradiated, shelf-life 24 hours to avoid hyperkalaemia
Rest of specification as for small-volume units

Source: Adapted from UK guidelines.

Washed red cells

Patients with recurrent or severe allergic or febrile reactions to red cells, and especially those with severe IgA deficiency with anti-IgA, may need washed red cells. These are produced in a closed, system in which the red cells are washed to remove most of the plasma and then resuspended in 100 mL SAG-M with a shelf life of 14 days after washing. Washed red cells can be irradiated at any time up to 14 days after collection. Irradiation should take place after washing. Following irradiation washed red cells that are suspended in a validated additive solution should be transfused as soon as possible and no later than 5 days after irradiation if irradiated on the day of washing, or 48 hours if irradiated after the day of washing.

Frozen red cells

Red cells can be stored for a prolonged period (up to 10 years) with minimal damage if glycerol is added before freezing. Thawed red cells must be washed free of glycerol before transfusion. This method of storage is expensive and time-consuming but is invaluable as a means of storing red cells with rare phenotypes (e.g. Bombay) and national banks of frozen rare cells have been established for this purpose.

Platelet preparations

Platelets are produced from whole blood (Figure 19.1) or by apheresis. Following the processing of whole blood, the buffy coat layer contains most of the platelets, over 80% of the white cells and 5–10% of the red cells. Four buffy coats are pooled together with platelet-additive solution (PAS). The plasma in the final component is made up of a small plasma content of each of the four buffy coats. The pool undergoes a second gentle centrifugation step and the supernatant is used to produce a platelet preparation, containing, on average, 3×10^{11} platelets. The residue from the buffy coat pool, containing mainly white cells and red cells, is then discarded. The systems that enable the separation of buffy coats in a semi-automated manner are called blood separators.

Unlike red cells, platelets must be stored at 20–22 °C, since storage at 4 °C results in poor survival following transfusion. Platelets have a shelf life of 5 days and are stored in gas permeable bags that allow the diffusion of oxygen into the pack, which, with constant gentle agitation, maintains aerobic metabolism and reduces the rate of fall of pH. The shelf life of platelet preparations can be extended to 7 days provided that systems are in place to monitor bacterial contamination (now used by the UK blood services) or to inactivate pathogens (not widely used at present).

The equivalent of two or even three adult doses of platelets (minimum 2.4×10^{11} each) may be obtained from one donor, with adequate platelet counts, by an apheresis procedure lasting approximately 90 minutes. A large HLA-typed donor panel is available in England to provide HLA-matched platelets for

immunologically refractory patients. Most apheresis platelet donors are also typed for human platelet antigens (HPA), in order to provide HPA-1a-negative platelets for newborns affected with neonatal alloimmune thrombocytopenia and also for a few HPA-1a-negative patients who have become immunologically refractory to platelet transfusions.

Platelets in additive solution (sometimes called 'washed platelets')

This component is indicated for patients with recurrent severe allergic or febrile reactions to standard platelet transfusions. After centrifugation and removal of most of the plasma, the platelets are resuspended in 200 mL of PAS but may still contain around 10 mL residual plasma.

Granulocyte concentrates

With the increase in intensive chemotherapy regimens, demand for granulocytes has risen in order to prevent or treat fungal or other serious, antimicrobial-resistant infections in severely neutropenic patients.

Granulocytes are extremely labile; they must be separated from whole blood immediately after collection and transfused within a maximum of 24 hours of preparation. Granulocytes prepared from routine blood donations (buffy coats) are heavily contaminated with red cells and platelets. Buffy coats from at least 10 donors are required to produce a therapeutic dose for an adult (at least 1×10^{10} granulocytes), or 10–20 mL/kg for children <50 kg. Ten buffy coats also contain the equivalent of two units of red cells and 2.5 pools of platelets, leading to reduction in transfusion requirements.

An optimised, pooled granulocyte component produced from whole blood donations is used in preference to buffy coats, when available. It has significantly less red cell contamination and provides double the number of granulocytes for a given volume (1×10^{10} per 250 mL pool; 2 pools for an adult therapeutic dose). Due to the overall high red cell content, granulocyte preparations should be ABO-compatible with the recipient and cross-match compatible by immediate spin against the recipient's plasma, if not eligible for electronic issue. Prospective recipients of granulocyte concentrates should be screened for HLA antibodies; if present, they can cause transfusion reactions, but more importantly, there is also increasing recognition that HLA-sensitisation impacts on engraftment and transplant success in mismatched allogeneic bone marrow transplant, particularly the increasingly used haplo-identical procedures.

All granulocyte concentrates must be irradiated prior to transfusion to reduce the Transfusion-associated Graft-versus-Host Disease (Ta-GvHD) risk. Other risks include febrile reactions and transfusion-related acute lung injury (TRALI).

Administration of granulocyte colony-stimulating factor (GCSF) or steroids can produce much higher yields of granulocytes collected by apheresis. This is not permitted for non-directed volunteer donors in the UK but is common in other blood services, particularly in the USA.

Importantly, the clinical effectiveness of granulocyte products remains a debated area, with published controlled trials producing conflicting results.

Fresh-frozen plasma (FFP)

Plasma that has been separated from whole blood (Figure 19.1) or obtained by apheresis is frozen within 24 hours to a temperature that will maintain the activity of the labile factors V and VIII. FFP contains all coagulation factors and FFP should be stored at a core temperature of –25 °C or below for a maximum of 36 months. Although a storage temperature below –25 °C improves the preservation of labile coagulation factors, lower temperatures increase the fragility of plastic. When needed, the plasma is thawed rapidly at 37 °C and then transfused without delay at a dose of 15 mL/kg in adults (see below).

Single units of FFP can be treated with photosensitising chemicals such as methylene blue (MB), riboflavin or amotosalen and exposed to visible or ultraviolet light to inactivate pathogens that may be present. If required, the residual chemicals can be removed before the plasma is rapidly frozen to −30 °C. Alternatively, FFP can be pooled with around 1500 other units and treated with solvent-detergent (SD) in order to inactivate pathogens. All methods of viral inactivation reduce the levels of labile clotting factors in FFP. This is considered to be a pharaceutical process with SD-FFP licensed as a medicinal product in the UK.

Following concerns about potential vCJD transmission in the UK, from 1998 until March 2019 FFP for use in patients born after 1996 was sourced from countries with a low risk of vCJD and pathogen-inactivated (MB or solvent detergent). Similarly, as a precautionary measure reversed in February 2021, UK plasma not used for FFP was discarded and not used for fractionation for the manufacture of immunoglobulins, albumin and coagulation factor concentrates. These decisions were reversed by the UK Advisory Committee on the Safety of Blood, Tissues and Organs (SaBTO) and the Commission on Human Medicines (CHM) respectively, following reviews of the safety of UK plasma and reduced concerns over the population risk of vCJD.

In recent years in the UK, every effort has been made to prepare FFP only from male donors in order to minimise the risk of passive transfer of donor white cell antibodies that can cause TRALI. PAS is used for suspension of pooled platelets.

Cryoprecipitate

Cryoprecipitate is prepared from FFP that is allowed to thaw slowly (classically at 4 °C overnight). After removal of the supernatant, factor VIII: C, von Willebrand factor (VWF), fibrinogen, fibronectin and factor XIII are left as a precipitate, which is then

refrozen in approximately 30 mL of plasma and stored at −30 °C or below for up to 24 months. Each unit should contain a minimum of 70 IU of factor VIII: C and 140 mg of fibrinogen. Cryoprecipitate is now used mainly as a source of fibrinogen in cases of major haemorrhage, disseminated intravascular coagulation (DIC), hepatic failure and hypofibrinogenaemia. A standard adult dose of cryoprecipitate is 10 units, which are thawed at 37 °C in about 10 minutes and should be used immediately. Pools of five single units are available in the UK. Fibrinogen concentrates are available in the UK with differing licences for use in congenital hypo-/a-fibrinogenaemia, and more recently in patients with acquired hypofibrinogenaemia and surgical haemorrhage.

Irradiated blood components

Patients at risk of transfusion-associated graft-versus-host disease (TA-GvHD, see further on) require irradiation of cellular blood components (red cells, platelets and granulocytes). For adults, red cells can be irradiated using gamma or X-rays within 14 days of donation, with a shelf life of 14 days from irradiation. Red cell components for exchange neonatal transfusion and intrauterine transfusion (IUT) should be irradiated within 5 days of donation with a post-irradiation shelf life of 24 hours. Platelets should also be irradiated to prevent TA-GvHD in susceptible patients and they retain their normal shelf-life post-irradiation. Frozen plasma components such as FFP and cryoprecipitate do not need irradiation.

Clinical and laboratory transfusion practice

In the UK, a series of three 'Better Blood Transfusion' Health Service circulars published in 1998, 2002 and 2007 promoted safe transfusion practices within hospitals, with emphasis on the appropriate use of blood and components in all clinical areas. Many of the principles of 'Better Blood Transfusion' are now encompassed in Patient Blood Management (PBM), an evidence-based, comprehensive multidisciplinary approach to the management of patients needing transfusion (see below).

All hospitals should have hospital transfusion committees (HTCs), with multidisciplinary representation from the clinical, laboratory and management areas. These committees are responsible for overseeing implementation of guidelines and for the audit and training of all staff involved in transfusion. The HTC is essential within the hospital clinical governance and must be accountable to the chief executive. The hospital transfusion team (HTT), composed of transfusion specialists with a nursing or biomedical background, the transfusion laboratory manager and the consultant haematologist specialising in transfusion, undertakes various activities on a day-to-day basis to achieve the objectives of the HTC.

EU blood directives (UK blood safety & quality regulations 2005): impact on hospital transfusion practice

The EU Blood Directives not only set standards for quality within the blood services, but also have had a far-reaching impact on hospital transfusion practice. In the UK, the chief executive of each hospital with a transfusion laboratory needs to submit a formal annual statement of compliance to the MHRA as the competent authority implementing the Blood Safety and Quality Regulations 2005. Hospital transfusion laboratories can be inspected by the MHRA and in the event of significant deficiencies be given the order to 'cease and desist from activities'. The key requirements for hospital transfusion laboratories include:

- a comprehensive quality management system based on the principles of good practice, including stringent requirements for storage and distribution of blood and components, with emphasis on 'cold chain' management;
- traceability, requiring all hospitals to trace the fate of each unit of blood/blood component (including name and patient ID) with records kept for 30 years;
- education and training of staff involved in blood transfusion, with maintenance of all training records;
- haemovigilance, with reporting of all adverse events of transfusion.

Hospital transfusion laboratories undertaking any processing activities, such as irradiation, must have a licence from the MHRA indicating they have a blood establishment status. NHSBT blood centres have such licences.

Laboratory tests in patients

Pretransfusion group and screen

The ABO and RhD groups of all potential recipients should be determined before transfusion, due to the universal presence of ABO antibodies in all subjects lacking the corresponding antigens and to the high immunogenicity of RhD (see Chapter 18).

The patient's red cells are grouped for ABO using monoclonal anti-A and anti-B. A 'reverse group' is carried out on the patient's plasma to demonstrate the presence of the corresponding antibody, using A and B reagent cells. RhD typing is undertaken using 2 IgM monoclonal anti-D reagents.

The patient's serum should also be screened for the presence of atypical red cell antibodies using the indirect antiglobulin test (IAT). If a positive result is obtained in this 2- or 3-cell screen, further investigation using a red cell panel of 8–10 cells is required to identify the antibody or antibodies and eventually provide antigen-negative cells for cross-matching. Depending on expertise in individual hospitals, antibody identification

may be carried out in-house or referred to red cell reference laboratories.

The majority of hospital laboratories in the UK carry out blood grouping and antibody screening using automated analysers with computer control of specimen identification and electronic transfer of results. This is safer than manual grouping techniques with manual transcription of results.

Since errors related to having the 'wrong blood in tube' (WBIT) are relatively common, with the potential risk of ABO-mismatched transfusions, the current British Society for Haematology (BSH) guideline recommends that a second sample should be requested for confirmation of the blood group of any new patient, provided this does not impede the delivery of urgent red cells or components, although there are numerous permutations by which other available historical blood group information might be acceptable. The exception to the 'two-sample rule' is the presence of a secure, electronic patient identification system; this significantly reduces the risk of WBIT although does not eradicate it completely. There is increasing adoption of this type of system within UK hospitals.

For most patients without clinically significant antibodies in their plasma, ABO- and RhD-compatible red cells are provided if transfusion is required. If the patient has clinically significant antibodies, the red cells provided must also be negative for the relevant red cell antigen(s), and serologically cross-matched before transfusion (see Chapter 18). Women of childbearing age should also receive K-negative units to avoid the risk of forming anti-K.

The BSH Compatibility Guidelines 2012 (updated 2017) provide pragmatic recommendations on the timing of pretransfusion samples; for the majority of patients this is up to 3 months, but testing should be performed using blood collected no more than 3 days in advance of the transfusion when the patient has been transfused or pregnant within the preceding 3 months. An extension to 7 days may be considered for regularly transfused patients and pregnant women with no significant alloantibodies who need blood on standby for a potential obstetric emergency such as placenta praevia.

Compatibility testing (cross-match)

Traditionally, a cross-match, by IAT at 37°C, between the patient's plasma and red cells from the donor unit has been the final step before blood is issued for transfusion. This still applies in specific cases (see below), but now there is no need for serological cross-match in many patients with no red cell alloantibodies, where the relevant laboratory requirements for safe electronic issue (EI) are in place.

Electronic cross-match/electronic issue (EI)

ABO- and RhD-compatible red cells can be provided using electronic issue (or 'computer cross-match') provided the patient does not have any alloantibodies and there are robust automated systems in place for blood grouping and antibody testing, interfaced with laboratory information systems to minimise risk of human error. If a patient has clinically significant red cell antibodies, EI should not be used and an IAT cross-match is required with antigen-negative red cell units. Electronic issue is also not suitable in certain patient groups, e.g. those with a history of an ABO-incompatible stem cell transplant or those who have had an ABO-incompatible solid organ transplant in the preceding 3 months.

In hospitals where electronic issue is not routinely used, a maximum (or standard) surgical blood ordering schedule (MSBOS, SBOS) can reduce the number of compatibility tests performed and avoid the reservation of red cell units unlikely to be transfused. The MSBOS is based on a retrospective comparison of the number of units of blood cross-matched and the number actually transfused, for each elective surgical procedure, at a given hospital. It should be agreed with the transfusion laboratory, surgeons and anaesthetists and ratified by the HTC. Procedures that are likely to require blood transfusion have a ratio of cross-matched:transfused below 2:1. Regardless of the MSBOS, if a typical antibodies are detected, antigen-negative blood should be cross-matched and reserved before surgery.

Special requirements for the selection of blood

Certain patient groups have special requirements regarding the selection of blood and components for transfusion. Clinical teams should be made aware of these requirements through the use of guidelines supported by education. Appropriate alerts for special requirements should be used within the transfusion laboratory. Serious Hazards of Transfusion (SHOT) haemovigilance reporting has highlighted the relative frequency of errors in this area of transfusion practice. The special requirements for intrauterine and neonatal transfusion are highlighted in Table 19.5.

Haemoglobinopathy

Patients with haemoglobinopathy, in particular those with sickle cell disease (SCD), are at a high risk of developing red cell alloantibodies, with reports of 20–30% in the literature (see also Chapters 14 and 15). Accordingly, from the start, patients with haemoglobinopathy should receive blood that has 'extended matching' for K and Rh antigens in addition to D, i.e. Cc Ee, in order to reduce the risk of alloimmunisation. Blood for transfusion to patients with SCD should also be HbS-negative. Blood for top-up transfusion in haemoglobinopathy patients should be

<2 weeks old, and <7 days old if used for exchange transfusion in patients with SCD.

In addition, the red cells of patients with SCD should be phenotyped extensively prior to transfusion for antigens apart from Rh and K such as for Kidd, Duffy and Ss antigens. Where patients have already been transfused, the genotype can be determined by molecular techniques. This information can assist with investigation of haemolytic transfusion reactions and expedite issue of blood in cases with complex serology. In addition, genotyping can identify variants, particularly in the Rh antigen group, which may have implications for red cell unit selection and complex antibody formation.

Haemato-oncology

Irradiation of cellular blood components (red cells, platelets, granulocytes) is required for certain groups of haematology patients, as highlighted in Table 19.6. Patients should be informed of the need for irradiated blood, supported by written information and cards flagging up their special requirements. Whilst CMV infection can be transmitted by cellular blood components and may produce fatal infection in immunocompromised patients, the risk can be reduced by leucodepletion (see previously). In haemato-oncology patients in the UK, there is no requirement to select for CMV-negative components apart from the rare circumstance of

Table 19.6 Indications for irradiated cellular blood components* in haemato-oncology patients.

Patient group	Irradiated blood components
Adults or children with acute leukaemia	Only for HLA-matched platelets, granulocytes or donations from first- or second-degree relatives, or if chemotherapy agent results in requirement.
Recipients of allogeneic (donor) HSC transplantation	From the start of conditioning chemo-radiotherapy, and continued until all the following criteria are met: >6 months have elapsed since the transplant date The lymphocyte count is >1·0 × 109/L The patient is free of active chronic GvHD The patient is off all immunosuppression If chronic GvHD or on immunosuppressive treatment, continue irradiated blood components
Bone marrow and peripheral blood stem cell donors	Provide irradiated cellular components during and for 7 days before the harvest
Bone marrow or peripheral blood stem cell harvesting for future autologous reinfusion	Provide irradiated cellular components during and for 7 days before the harvest
Autologous stem cell transplant patients	From start of conditioning chemo-radiotherapy until 3 months post-transplant (6 months if total body irradiation was used)
Adults and children with Hodgkin lymphoma at any stage of the disease	Irradiated cellular components indefinitely
Patients treated with purine analogues and related compounds (fludarabine, cladribine pentostatin, bendamustine)	Irradiated cellular components indefinitely
Patients treated with alemtuzumab (anti-CD52) or anti-thymocyte globulin (ATG) therapy**	Irradiated cellular components indefinitely
Chimeric antigen receptor T-cell (CAR-T) therapy	For 7 days prior to and during peripheral blood lymphocyte collections (in autologous CAR-T procedures) for future CAR-T cell reinfusion. Continue to be used until 3 months following CAR-T cell infusion unless conditioning, disease or previous treatment determines indefinite duration
Rituximab / anti-CD20	Not indicated as a sole reason

*Red cells, platelets and granulocytes.
**Irradiated components are now not recommended for solid organ transplant, vasculitis or multiple sclerosis patients receiving alemtuzumab or ATG at currently used dose schedules.
Source: Based on BSH guidelines 2020.

Table 19.7 Selection of ABO blood group of components transfused in the early* post-transplant period following allogeneic bone marrow transplant.

	Donor	Recipient	Red cells	Platelets	FFP
Major ABO incompatibility	A	O	O	A	A
	B	O	O	B	B
	AB	O	O	A	AB
	AB	A	A/O	A	AB
	AB	B	B/O	B	AB
Minor ABO incompatibility	O	A	O	A	A
	O	B	O	B	B
	O	AB	O	A	AB
	A	AB	A/O	A	AB
	B	AB	B/O	B	AB
Bidirectional ABO incompatibility	A	B	O	B	AB
	B	A	O	A	AB

* Once conversion to donor blood group is complete, components of the new ABO group can be given.
Source: Adapted with permission from Handbook of Transfusion Medicine 2014.

granulocytes given to CMV-negative allogeneic bone marrow transplant recipients.

Patients undergoing allogeneic stem cell transplantation may have a mismatch of ABO groups with their donor (perhaps up to 25% of HLA-identical sibling transplants) with a risk of haemolysis. There can be major ABO incompatibility whereby the recipient's plasma contains anti-A, anti-B or anti-A, B incompatible with donor red cells (e.g. group A donor and group O recipient), minor ABO incompatibility where the donor's plasma contains anti-A, anti-B or anti-A, B that can react with the recipient's red cells (e.g. donor group O and recipient group A) or bidirectional ABO incompatibility where both the donor and recipient's plasma contain anti-A, anti-B or anti-A, B reactive with recipient and donor red cells respectively (e.g. donor group A and recipient group B). Clear communication is essential between the clinical and laboratory teams, with an agreed post-transplant transfusion policy (see Table 19.7).

Antenatal testing

All women should have blood samples taken at antenatal booking for blood grouping and antibody screening. If no clinically significant antibody is detected, further samples should be tested at 28 weeks' gestation, before routine antenatal anti-D prophylaxis is given to RhD-negative women (see below).

Neonatal 'top-up' transfusion

Premature infants are amongst the most widely transfused patients, with 'top-up' transfusions being very frequent (Chapter 54). During the first 4 months of life, ABO antigens may be poorly expressed on red cells and the corresponding ABO antibodies may not have yet developed so that results of 'reverse grouping' will be unreliable. Passive transfer of maternal IgG ABO antibodies may result in the detection of these antibodies in neonatal plasma. Samples from both the mother and infant should be tested for ABO and RhD grouping wherever possible, with an antibody screen performed on the maternal sample. If there are no atypical maternal antibodies, top-up transfusions can be given without further testing during the first 4 months of life.

Ideally, the unit of red cells used for the first transfusion should be aliquoted into several (six to eight) satellite (or 'paedi') packs and used for the same infant until expiry in order to decrease exposure to multiple donors. Measures should be in place in all neonatal units and their supporting laboratories to minimise the quantities of blood required for testing, by use of micro-sampling techniques and near-patient testing, since frequent blood sampling significantly contributes to anaemia in these infants. Erythropoietin has been extensively studied as an alternative to transfusion in the anaemia of prematurity, but it does not consistently reduce the need for transfusion in these patients.

If a neonatal patient has received a prior IUT, any top-up transfusions must be irradiated.

Transfusion in autoimmune haemolytic anaemia

Patients with autoimmune haemolytic anaemia (AIHA) pose a significant challenge (see also Chapter 17). Whilst unnecessary transfusion should be avoided, this must not be withheld in the context of brisk haemolysis or where there is clinical concern such as risk of cardiac compromise. Close liaison between clinical teams, the transfusion laboratory and the specialist

immuno-haematology team within blood services is essential to assist with prompt investigation and issue of blood. These patients often have pan-reactive autoantibodies that can mask an underlying alloantibody, requiring investigation with serological techniques such as elution and absorption. Blood should then be selected that is ABO-compatible, matched with the patient's own full Rh and K type and negative for the antigens to which alloantibodies have been identified, if any. Avoidance of the specificity of the autoantibody (e.g. auto-anti-e in RhD-negative female patients) is not routinely recommended since this may expose the patients to antigens provoking the formation of clinically significant alloantibodies. Transfused red cells are likely to be destroyed by the autoantibody at the same rate as the patient's own blood. Hence, transfusion, if needed in AIHA, will serve only as a temporary 'holding measure' and should be accompanied by effective immunosuppressive therapy.

Safe administration of blood

The principles underpinning safe bedside transfusion practice should be encapsulated in a hospital transfusion policy covering aspects of requesting, collecting and administering blood components, as well as the monitoring of the patient during transfusion. It is imperative that staff involved in any part of the transfusion process maintain competence in the relevant skills, and responsibility for overseeing this competence assessment should lie with the HTT and the associated clinical governance procedures.

Patient identification

Positive patient identification (ID) is essential at all stages of the transfusion process, including blood sampling, collection of blood from storage, delivery to the clinical area and administration. Patients should have an identification band with the minimum identifiers, including their last name, first name, date of birth and unique identification number (e.g. hospital number). Many hospitals are investing in secure electronic patient identification systems which can replace the requirement for written/printed identification bands with digitally recognised wristbands. Electronic patient ID systems have been shown to reduce the number of 'wrong blood in tube' errors (WBITs) and wrong components transfused.

WBIT errors can result in death. These occur due to human error either when the blood in the tube is from the wrong patient and is labelled with the intended patient's details, or blood is taken from the intended patient, but the tube is labelled with the wrong details. It is estimated that this occurs in 1 in every 2000 samples taken. The SHOT UK haemovigilance scheme continues to report significant numbers of WBIT errors with 673 cases in 2020; these are mostly 'near-miss' events which could have resulted in the wrong blood being transfused. Nearly two-thirds of these events involve serial errors in the identification process rather than isolated ones, meaning there was more than one opportunity to identify the mistake. SHOT estimates that approximately 1 incorrect blood component is transfused for every 100 near-miss events.

Detection and approach to transfusion reactions

All patients should be transfused in clinical areas where they can be directly observed to allow prompt detection and immediate management of acute reactions. Baseline observations and regular monitoring of patients undergoing transfusion are essential so that reactions are detected and promptly managed. Patients with severe transfusion reactions can present suddenly with cardiovascular collapse, but the underlying cause may not be immediately apparent; these can include bacterial contamination, acute haemolytic transfusion reactions (AHTRs, usually due to ABO-incompatible transfusion), anaphylaxis, TRALI and transfusion-associated circulatory overload (TACO). If the presumed transfusion reaction is severe or life-threatening, the transfusion must be discontinued, with immediate assessment and the following steps taken:

1 stop and disconnect the blood pack and giving set immediately (but do not discard);
2 maintain venous access with physiological saline, commence resuscitation, if needed;
3 take samples for full blood count, renal and liver function tests, blood cultures, coagulation screen, repeat compatibility testing, DAT, LDH and assessment of urine for haemoglobin;
4 check identification details for the patient, their identity band and the blood component/s concerned;
5 consider key/additional features, e.g. fever and shock without anaphylaxis – consider ABO-mismatched transfusion or bacterial sepsis; dyspnoea – consider TRALI or TACO and check O_2 saturations or blood gases and chest x-ray; laryngeal oedema with dyspnoea/stridor – consider anaphylaxis or severe allergic reaction;
6 seek early support and advice from the critical care team.

Less severe acute transfusion reactions include haemolytic transfusion reactions due to other red cell antibodies in the recipient, FNHTR and allergic reactions. Whilst the latter two can often be managed with an antipyretic or antihistamine, respectively, ongoing close monitoring of the patient is essential, since these could be the early signs of a more severe transfusion reaction.

Complications of blood transfusion

The complications of blood transfusion can be conveniently divided into acute and delayed, immunological and non-immunological categories (Table 19.8).

Table 19.8 Hazards of transfusion.

Immediate (hours)
Non-immune complications
Bacterial: acute sepsis or endotoxic shock
Hypothermia
Hypocalcaemia in infants and in the context of massive transfusion
Transfusion-associated circulatory overload (TACO)
Immune complications
Febrile non-haemolytic transfusion reactions
Acute haemolytic transfusion reactions: intravascular (IgM), extravascular (IgG)
Allergic reactions
Anaphylactic reactions
Transfusion-related acute lung injury (TRALI)
Delayed (days to years)
Non-immune complications
HIV, HCV, HBV, CMV
Others: parvovirus B19, HAV, HEV, WNV, dengue, malaria, Chagas disease, brucellosis, syphilis, vCJD
Immune complications
Delayed haemolytic transfusion reactions (due to anamnestic immune responses with red cell alloantibodies)
Post-transfusion purpura
Transfusion-associated graft-versus-host disease

CMV, cytomegalovirus; HAV, hepatitis A virus; HEV, hepatitis E virus; WNV, West Nile virus.

Sensitisation to red cell antigens

As only the ABO and RhD antigens are routinely matched for the selection of blood, sensitisation can occur to other red cell antigens following transfusion. Although the D antigen is the most immunogenic, other antibodies can form with alloimmunisation being more likely in multi-transfused patients. Red cell antibodies can also develop during and following pregnancy. The consequences of sensitisation may be negligible but can lead to difficulty with compatibility testing, haemolytic disease of the foetus and newborn (HDFN) and haemolytic transfusion reactions (HTRs) in the future.

Haemolytic transfusion reactions

This is premature destruction of transfused red cells reacting with antibodies in the recipient. Naturally occurring antibodies, such as ABO antibodies, are mainly IgM and, if warm-reacting, can destroy red cells *in vivo* by complement fixation. Red cell alloantibodies in response to exposure through previous transfusions or pregnancies are usually IgG antibodies. HTRs may occur immediately after the transfusion or may be delayed for anything up to 2–3 weeks.

Immediate or acute haemolytic transfusion reactions

These may be intravascular or extravascular (Table 19.9).

Immediate intravascular red cell destruction is the most dangerous type of HTR; it is associated with activation of the full complement cascade by IgM antibodies and is practically always due to ABO-incompatible blood transfusions (haemolytic anti-A, B, anti-A or anti-B present mainly in the recipient or, rarely, in the donor plasma). Most of these ABO-incompatible transfusions are due to identification errors and occur with an approximate frequency of 1 in 220,000 patients transfused in the UK based on data from 2020. Major morbidity with patients needing intensive care or renal dialysis occurs in 20–25% of all cases with a mortality rate of 5–10%. In recipients (mostly group O) of ABO-incompatible blood with serious reactions, the symptoms are usually dramatic and severe; most are due to the anaphylatoxins C3a and C5a that are liberated during complement activation, releasing vasoactive amines and hydrolases from mast cells and granulocytes. The cytokines interleukin (IL)-1, IL-8 and tumour necrosis factor (TNF) also play an important role, causing inflammation, smooth muscle contraction, platelet aggregation, increased capillary permeability and hypotension. Typically, within less than 1 hour of the start of the transfusion, when the reaction is symptomatic, the patient complains of heat or pain in the cannulated vein, throbbing in the head, flushing of the face, chest tightness, nausea and lumbar

Table 19.9 Antibodies associated with haemolytic transfusion reactions.

Blood group system	Antibodies implicated in intravascular haemolysis	Antibodies implicated in extravascular haemolysis
ABO	-A,B; -A; -B	
Hh	-H (Bombay)	
Rh		All
Kell		K, k, Kp^a, Kp^b, Js^a, Js^b
Kidd		Jk^a, Jk^b, Jk^3
Duffy		Fy^a, Fy^b, Fy^3
MNS		M, S, s, U (some)
Lutheran		Lu^b (some)
Lewis	Le^a, Le^b, Le^{a+b}	
Cartwright		Yt^a (some)
Vel	Vel	Vel (some)
Colton		Co^a, Co^b
Dombrock		Do^a, Do^b

pain. These symptoms are usually accompanied by tachycardia and hypotension. In severe cases, there is profound hypotension, leading to shock and renal failure. Rigours and pyrexia usually follow.

Intravascular destruction of red cells also brings about liberation of thromboplastin-like substances that activate coagulation and lead to DIC. The bleeding diathesis and increased destruction of red cells (which may eventually involve the recipient's cells) further exacerbate the problem. Intravascular destruction of red cells liberates haemoglobin into the circulation. Once haptoglobins are saturated, haemoglobin will also appear in the urine. If haemoglobinuria is very severe, haemosiderinuria may be seen. Renal complications consist of acute kidney injury with oliguria and anuria, possibly the result of hypotension and/or the action of activated complement. The initial symptoms may of course be modified or abolished in anaesthetised or heavily sedated patients, in whom evidence of DIC, hypotension or the presence of haemoglobinuria may be the first signs.

The most severe reactions occur in major incompatibility, when a group O recipient with high-titre anti-A, B is transfused with group A, B or AB red cells. Less severe intravascular haemolysis occurs when group A red cells are transfused to a group B recipient, or vice versa, because group B and A subjects have less potent ABO antibodies than group O subjects. More rarely, intravascular red cell destruction may occur when group O plasma is transfused by mistake to A, B or AB recipients. For this reason, group O red cells, even in additive solution, should not routinely be used for non-O recipients; furthermore, this practice leads to unnecessary shortages of group O blood. If unavoidable, the group O blood must first be screened for the presence of high-titre haemolysins or should have very small volumes of plasma. ABO-compatible cryoprecipitate, FFP and platelet transfusions should be selected for all recipients, especially for children because of their smaller blood volume. Group A, B or AB plasma components are safe for group O recipients.

There is also the possibility of a laboratory error when tubes are transposed or there are transcription errors or mis-recording of results, or when there is insufficient time to complete an antibody screen or compatibility test.

In the UK haemovigilance (SHOT) system, approximately 55% of cases of incorrect blood component transfusion reported in 2020 ($n = 323$) have been primarily due to clerical or technical errors that originated in the hospital laboratory. Obviously, not all the errors led to ABO-incompatible transfusions; in fact, 5% of errors led to ABO incompatibilities. The remainder of reports (45%) relate to clerical or administrative errors in the ward, collection of the blood from the blood bank, failure to confirm the identity of the patient when taking samples, mislabelling of the sample of blood or failure to perform proper checks before removing the units from the refrigerator or transfusing the blood. The potentially serious consequences of failures resulting in ABO-incompatible transfusions emphasise the need for set protocols with meticulous checking at all stages. If an identification mistake has been made, it is important to check, as a matter of urgency, that the units intended for the patient under investigation have not also been misdirected to another recipient. Such events are results of errors that need further investigation with a root cause analysis so that corrective action can be taken to minimise recurrence.

Extravascular red cell destruction is mediated by IgG antibodies (Figure 19.3). Mononuclear phagocytic cells have receptors for the Fc fragment of IgG1 and IgG3; the binding of IgG-coated cells to these receptors is inhibited by free IgG in plasma. There are no receptors for IgM on macrophages. Red cells sensitised with IgG1 and/or IgG3 antibodies may or may not activate complement up to C3b only. If they do not, they are removed extravascularly (by phagocytosis or cytotoxicity) by mononuclear phagocytic cells, predominantly in the red pulp of the spleen, where the plasma is largely excluded and the IgG on the red cells can compete with free IgG in the plasma. However, cells coated with IgG antibodies, which activate complement up to C3b, adhere to the C3b

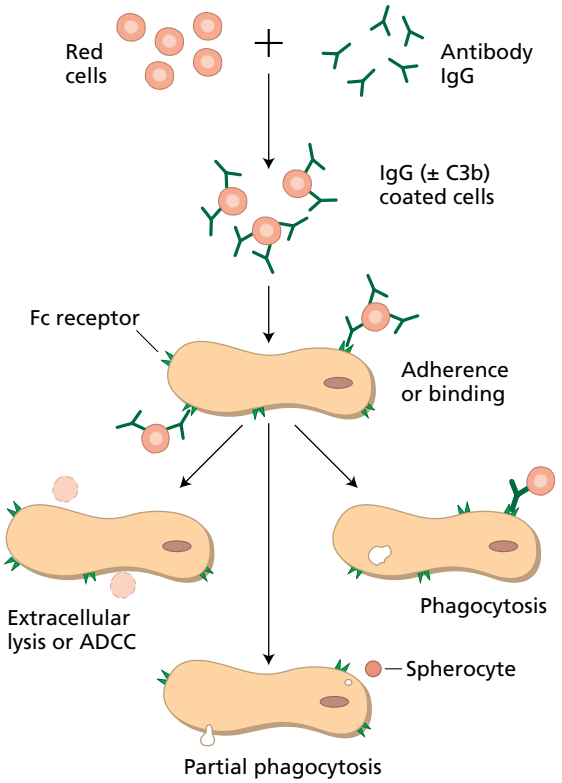

Figure 19.3 Mechanisms of extravascular destruction of red cells coated with IgG1 or IgG3 (± C3b). ADCC, antibody-dependent cell-mediated cytotoxicity.

receptor on macrophages and monocytes. The presence of C3b on red cells greatly enhances the extravascular destruction of IgG-coated cells. This is because the binding to C3b receptors is not inhibited as there is no native C3b in plasma and consequently IgG/C3b-coated cells are destroyed by phagocytosis or cytotoxicity, predominantly in the liver, where there are abundant macrophages (Kupffer cells) and a generous blood flow. As C3b is rapidly inactivated and converted into C3dg by the action of factors H and I and proteases, a proportion of the cells re-enter the circulation, coated with C3dg and are resistant to further lysis (Figure 19.3). Red cells coated with potent IgG antibodies, especially if they are C3b-binding, are destroyed mainly by cytotoxicity. Red cells coated with less potent antibodies are mostly destroyed by phagocytosis. Very rarely, red cell alloantibodies too weak to be detectable by routine pretransfusion testing may destroy donor red blood cells carrying the corresponding antigen.

The features of an immediate HTR vary according to a number of factors: whether the red cells are destroyed within the circulation or in the mononuclear phagocytic system, the strength, class and subclass of the antibody, the nature of the antigen, the number of incompatible red cells transfused and the clinical state of the patient. When antibodies are present in the circulation in low titres and a large volume of incompatible blood is given, all circulating antibodies will bind to the incompatible red cells, coating them weakly, without destroying them. There will then be no antibody detectable in the serum for a number of days until secondary antibody production is stimulated by the immune challenge. On the other hand, in the presence of an overloaded or poorly functioning mononuclear phagocytic system, large volumes of IgG-sensitised incompatible red cells can be present in the circulation with minimal or no premature removal, so the haemoglobin level may be stable with little evidence of haemolysis. The DAT will be positive, but as there is no free antibody, elution techniques will be necessary for antibody identification.

Immediate extravascular destruction of red cells may be accompanied by hyperbilirubinaemia, occasionally haemoglobinaemia due to antibody-dependent cytotoxicity (in severe cases), fever and failure to achieve the expected rise in haemoglobin level. The signs and symptoms are less severe and dramatic than in intravascular haemolysis and usually appear more than 1 hour after the start of transfusion. There may be no signs or symptoms at all. Acute kidney injury is very rare, even when the antibody binds the earlier components of the complement cascade. Symptoms are attributed in large degree to liberation of cytokines from mononuclear phagocytic cells after binding to IgG-coated red cells and to liberation of C3a when complement is bound up to C3b. The mortality is extremely low, but in an already sick patient the added complication of destruction of transfused red cells may contribute to death.

Table 19.10 Investigation of suspected acute haemolytic transfusion reaction.

Blood test	Rationale/findings
Full blood count	Baseline parameters, red cell agglutinates on film
Plasma/urinary Hb, haptoglobin, bilirubin	Evidence of intravascular or extravascular haemolysis
Blood group of patient and units transfused	Compare with retested pretransfusion sample, to detect ABO error Unexpected ABO antibodies may arise from transfusion of incompatible plasma. Re-checking labels is often sufficient
DAT	Positive in majority. Compare with retested pretransfusion sample. May be negative if all incompatible cells destroyed; eluate must still be checked in these cases.
Compatibility testing	Repeat antibody screen and compatibility testing on pre- and post-transfusion samples. Elution of antibody from post-transfusion cells may aid antibody identification or confirm specificities in plasma in cases of non-ABO incompatibility
Urea, electrolytes and creatinine	Baseline renal function
Coagulation screen	Detection and monitoring of DIC
Blood cultures of patient and units	In event of possible septic reaction caused by bacterial contamination of unit

The management of acute HTRs should be to terminate the transfusion as soon as the patient develops relevant signs or symptoms. The identity of the patient and the units transfused should be checked against the appropriate documentation. Blood samples must be taken for investigation (Table 19.10). The transfusion laboratory should be immediately informed and all packs of transfused units should be returned. Pretransfusion samples should be tested in parallel. All urine passed during the first 24 hours should be measured and examined for haemoglobin. The circulating blood volume should be restored and blood pressure and urinary flow maintained using fluid challenges and furosemide. Monitoring on a high-dependency unit may be required. The renal team should be involved early if urine output is poor (<1 mL/kg/hour) and haemofiltration may be necessary. Appropriate blood component therapy may be required if there is DIC.

Delayed haemolytic transfusion reactions (DHTRs)

Such reactions may not be predictable or preventable. They are always caused by IgG antibodies; hence, the haemolysis is always extravascular. In the majority of cases, an individual has been previously sensitised to one (or more) red cell antigen(s) by previous transfusion, pregnancy or transplant. Antibody is not detectable in routine pretransfusion testing, but the transfusion of blood containing the antigen/s to which the recipient has been sensitised previously, provokes a brisk anamnestic response that is characteristic of the secondary immune response. Within days, the antibody level rises and the transfused incompatible cells are removed from the circulation. The effects of the secondary immunisation are usually seen about 5–10 days after the transfusion, when the antibody level has been boosted and the recipient may already have left hospital. The clinical features are the same, though less severe than in immediate extravascular HTR.

The possibility of delayed HTRs underlines the importance of always taking fresh serum samples for antibody screening, direct antiglobulin test (DAT) and compatibility testing if a transfusion has been given more than 72 hours previously. Awareness of this complication may avoid unnecessary investigations to exclude infection when fever develops a few days after a transfusion. Most importantly, it will detect any alloantibody that has been boosted by the transfusion, thus enabling the provision of compatible blood. Similarly, it is important to have access to historical transfusion information since antibodies previously formed (e.g. Kidd antibodies) may become undetectable with time and may not be apparent in the current antibody screen. This is particularly important if patients are managed at more than one hospital, and highlights the continuing importance of improved inter-laboratory IT connectivity and data-sharing.

Laboratory investigations of suspected DHTRs include full blood count and reticulocytes, examination of the blood film, plasma bilirubin, renal function tests and lactate dehydrogenase test (LDH). Serological investigations should include repeat blood group and antibody screen (on pre- and post-transfusion patient samples), DAT and elution of antibodies from the post-transfusion red cells for identification.

Treatment of DHTRs is usually supportive, sometimes requiring further transfusion. The presence of any antibodies should be documented within the laboratory and clinical records, with patients issued with an 'Antibody Card'.

Febrile non-haemolytic transfusion reactions (FNHTRs)

Febrile reactions are most frequently due to the transfusion of blood components containing white cells to patients sensitised to white cell antigens. Antibodies are usually against HLA antigens, or sometimes against granulocyte and platelet-specific antigens; they are stimulated by previous transfusions or pregnancies. Cytokines released from white cells during storage may also be pyrogenic. These reactions are characterised by fever, sometimes accompanied by shivering, muscle pain and nausea, but are much less common since the introduction of leucodepletion of blood components. They can occur up to 2 hours after completion of the transfusion and are more common in multi-transfused patients receiving red cells.

Mild FNHTRs (pyrexia >38 °C, but <2 °C rise from baseline) can often be managed simply by slowing (or temporarily stopping) the transfusion and giving an antipyretic, such as paracetamol. However, the patient should be monitored closely, in case these are the early signs of a more severe transfusion reaction.

Transfusion-related acute lung injury (TRALI)

TRALI is non-cardiogenic pulmonary oedema, presenting during or within 6 hours of transfusion, with severe breathlessness accompanied by cough, fever and rigours. It is often associated with hypotension (due to loss of plasma volume) and there may be transient peripheral blood neutropenia or monocytopenia. Chest X-ray shows bilateral nodular shadowing in the lung fields with normal heart size, low oxygen saturation and low or normal central venous pressure. TRALI may be confused with acute respiratory distress syndrome (ARDS) of other causes, or with acute heart failure due to circulatory overload (see Table 19.11), but treatment with diuretics may worsen the outcome. Management is essentially supportive, requiring care in a high-dependency unit with ventilatory support, if needed. Steroid therapy is not effective. Managed appropriately, there is a high rate of survival and most patients recover within 1–3 days without long-term sequelae.

The reaction has been defined as due to passive transfer of leucoagglutinins (mostly anti-HLA class I or class II or, more rarely, granulocyte antibodies, i.e. anti-HNA) in donor plasma, reacting with granulocytes in the recipient's lungs, leading to complement activation, endothelial and epithelial injury, alveolar damage and inflammatory changes, mediated by anaphylatoxins, cytokines and other inflammatory mediators. This complication is more likely after infusion of a plasma-containing component and the implicated donors are usually multiparous women. In the UK, the incidence of classical antibody-mediated TRALI has declined to approximately 1 in one million units transfused, since the introduction of the policy to produce FFP and plasma to suspend platelet pools mainly from male donors.

In 2019 a true redefinition of the TRALI diagnosis was proposed by an international working group, requiring only a constellation of clinical features and no contributing immunological findings. This awaits further validation, but from the perspective of blood services, a definition that enables reduction of TRALI risk through amelioration of a well-documented immunological risk is advantageous.

Table 19.11 Comparison of transfusion-related acute lung injury (TRALI) and transfusion-associated circulatory overload (TACO).

	TRALI	TACO
Patient characteristics	More frequently reported in haematology and surgical patients. Any age	Most common in age >70, but can occur at any age
Implicated blood components	Usually plasma or platelets	Any
Onset	Up to 6 hours from transfusion (usually within 2 hours)	Within 6 hours of transfusion
Oxygen saturation	Reduced	Reduced
Blood pressure	Often low	Often high
Jugular venous pressure	Normal or low	Elevated
Temperature	Often raised	Normal
Chest X-ray	Bilateral peri-hilar and nodular shadowing or 'white out', heart size normal	Enlarged heart and characteristics of pulmonary oedema
Echocardiogram	Normal	Abnormal
Pulmonary artery wedge pressure	Normal	Elevated
Blood count	Fall in neutrophils and monocytes followed by neutrophil leucocytosis	No specific changes
Fluid challenge	Improves	Worsens
Response to diuretics	Worsens	Improves

Source: Adapted from BSH Guideline on the Investigation and Management of Acute Transfusion Reactions, 2012, with permission of British Society of Haematology.

Transfusion-associated graft-versus-host disease (TA-GvHD)

TA-GvHD is a very rare, but usually fatal, complication of blood transfusion. It is caused by transfusion of viable donor lymphocytes with engraftment and clonal expansion of HLA-compatible donor T-lymphocytes in the recipient. It is characterised by fever, skin rash, diarrhoea, impaired liver function and pancytopenia 7–14 days after transfusion. Patients at risk include foetuses receiving intrauterine transfusion, newborn infants who have received IUTs, some patients with inherited immunodeficiency disorders affecting T-cell function, and patients treated with allogeneic stem cell transplantation or specific chemotherapy drugs such as purine analogues. TA-GvHD has occasionally been reported in non-immunosuppressed patients receiving a blood transfusion from an HLA-matched donor or a close relative with HLA types in common; accordingly all directed donations must be irradiated. Patients receiving conventional combination chemotherapy for cancer are not at increased risk.

The diagnosis is made on the basis of typical features of acute GvHD in biopsies of affected organs, with detection of donor-derived cells or DNA in the patient's blood or tissues. The first SHOT reports in 1996 included 13 cases of Transfusion-associated Graft-versus-Host disease, all fatal, in patients for whom gamma-irradiated blood components were not indicated at the time. Only one case of TA-GvHD has been reported in the UK since 2000 (an IUT of non-irradiated maternal blood). Given the potential fatal outcome of this complication of transfusion, it is essential to ensure that all at-risk patients receive irradiated red cells or platelet components.

Post-transfusion purpura (PTP)

PTP is a rare complication of blood transfusion, characterised by sudden onset of severe thrombocytopenia 7–10 days following transfusion of, usually, red cells. The patients are mostly female, always with a history of previous blood transfusions or pregnancies. The most frequent cause is the presence in the recipient of an antibody (anti-HPA-1a) against the platelet-specific antigen HPA-1a (PI^{A1}); next in frequency is anti-HPA-5b. Women with foetal and neonatal alloimmune thrombocytopenia (FNAIT) pregnancies are therefore at risk. It appears that the antigen–antibody reaction causes both transfused and autologous platelets to be prematurely destroyed, either by the formation of immune complexes (in a manner similar to the 'innocent bystander' mechanism) or by cross-reaction of the causative antibody with the patient's own platelets.

The disease is serious and frequently associated with severe bleeding, meaning prompt therapy with high-dose intravenous immunoglobulin (e.g. 2 g/kg over 2–5 days) should be implemented as soon as a clinical diagnosis is made and without

waiting for the results of investigations. This is associated with a response in about 85% of patients. Platelet transfusion is not recommended and should only be considered in life-threatening bleeding. This severe, and potentially fatal, complication has become even rarer since the introduction of leucodepleted blood components.

Mild allergic reactions

Mild urticarial reactions without other symptoms are not uncommon during blood transfusion; they occur with an approximate incidence of 1% and are mediated by IgE antibodies, usually against plasma proteins or other allergens present in donor plasma. Symptoms include urticaria (hives) and pruritus but with no change in vital signs. They are most common in patients receiving plasma-rich components such as FFP or platelets.

Mild urticarial reactions may be treated effectively with antihistamines and do not always recur. Standard components can be given unless symptoms are recurrent and severe.

Severe anaphylactic reactions

Anaphylaxis is an acute, life-threatening emergency associated with shock or severe hypotension with associated features including bronchospasm, stridor from laryngeal oedema and swelling of face, limbs or mucous membranes (angioedema), although this may be absent in up to 20% of cases. Components with a high plasma component such as platelets or FFP are most likely to be implicated, but such reactions may occur with all blood components, as they all contain some plasma. Staff in clinical areas carrying out blood transfusions must be trained in the emergency management of anaphylaxis, and epinephrine must be available for emergency use. The UK Resuscitation Council Guidelines recommend the urgent administration of intramuscular (IM) adrenaline to treat anaphylaxis (see latest guidelines for dosing).

Although IgA deficiency is relatively common and may occur in up to 1 in 700 individuals tested, only a small minority of IgA-deficient patients are at risk of developing severe anaphylactic reactions to blood components. Those with severe IgA deficiency (<0.07 g/L), with anti-IgA are at greatest risk. Patients with less severe IgA deficiency, as part of a more generalised antibody deficiency disorder (e.g. common variable immunodeficiency or secondary to a lymphoproliferative disorder) and mild cases frequently detected when screening for IgA coeliac antibodies, are not at risk. Patients with no history of severe reactions to blood transfusion should be transfused with standard blood components. Accordingly, it is the small minority of patients with severe IgA deficiency and a clear history of serious anaphylactic reaction to blood components that require special precaution and, if possible, transfusion of blood components from IgA-deficient donors. However, this might be feasible only in the elective setting, in countries with well-established blood services. In an urgent situation, washed red cells and platelets resuspended in platelet-additive solution may be available, but in an extreme emergency, transfusion with standard blood components should not be withheld, but given with careful monitoring to allow timely detection and treatment of the anaphylactic reaction, as above.

Transfusion-transmitted infection

Bacterial contamination of blood components (see also section above under TTI)

This complication can rapidly be fatal and may occur particularly with platelet components, which are stored at 20–24 °C, rather than with red cells, which are refrigerated at 2–6 °C. These reactions can either be due to the septicaemia itself, to endotoxins or both. The patient can present dramatically with collapse, high fever, shock and DIC.

Various measures to reduce bacterial contamination from the donor arm as highlighted above have significantly reduced this risk, but awareness and prompt detection and management remain essential. It is important to note that only platelet donations are routinely screened for the presence of bacteria because they are stored at 22 °C – a temperature which may encourage the growth of bacteria. Following screening, platelets have a shelf life of up to 7 days and any platelet packs reactive on screening are removed from the supply and further tested. Most bacteria detected on screening are unlikely to cause harm, but sometimes more significant bacteria are detected such as those normally found in the gut. Donors are contacted if their platelet donation grows bacteria which may indicate an underlying illness, where appropriate donors will be referred to their physicians for further investigations.

Bacterial screening is one of several strategies introduced to reduce the risk of bacterial TTIs, though this cannot guarantee the absence of contamination: packs are released for issue as 'negative-to-date', which may be before bacteria have multiplied sufficiently to trigger detection on screening. Bacterial screening of platelets became universal in the UK in 2011; since then, there has been one confirmed bacterial TTI, due to Staphylococcus aureus, in 2015. There have been ten bacterial near misses, all but one in platelet components, reported in the UK between 2011 and 2020. Overall, out of a total of 44 bacterial transfusion transmissions to individual recipients, 37 (34 donations) have been caused by the transfusion of platelets, and 7 by red cells in the UK since reporting began in 1996.

The immediate management and investigation in any suspected case of a transfusion recipient follow the principles as outlined above. In particular, the pack should be inspected for abnormal discolouration or aggregates but may appear normal. Blood cultures should be taken from the patient and immediate treatment started for shock, as appropriate and with intravenous

broad-spectrum antibiotics, covering Gram-negative and Gram-positive bacteria. Any packs of implicated components must be returned to the transfusion laboratory for instigation of further testing including Giemsa staining for direct microscopic examination, as well as culture. The Blood Service should be informed without delay so that any associated components from the implicated donation can be identified and withdrawn before they can be transfused to other patients. In the UK, the implicated component packs should be returned to the Blood Service for investigation, where comprehensive bacterial testing is undertaken together with typing of strains to confirm the identity of contaminating bacteria.

Transfusion-associated circulatory overload (TACO)

TACO is defined as acute or worsening respiratory compromise and/or acute or worsening pulmonary oedema during or up to 12 hours (SHOT accepts cases up to 24 hours) of transfusion, with additional features including cardiovascular system changes not explained by the patient's underlying medical condition, evidence of fluid overload, and a relevant biomarker. A new surveillance definition for TACO was published in 2019 which facilitates more consistent reporting of TACO (Table 19.12).

TACO requires the presence of at least one 'required' criterion within 12 hours of transfusion, plus a total of three or more other criteria (either required or additional):

Serum brain natriuretic peptide (BNP) or NT-pro brain natriuretic peptide (NT-Pro BNP) are potentially useful investigations in supporting a diagnosis of TACO and can often be performed on the same sample type as that used for compatibility testing, allowing convenient retrospective testing of pre- and post-transfusion blood samples. Of note, the other 'additional' parameters shown for the diagnosis of TACO have their limitations, since changes in cardiovascular status may be non-specific and can occur in both TRALI and TACO; hypotension is more commonly seen in TRALI. Fever has been shown to be present in both TACO and TRALI in approximately 30% of TACO cases suggesting an inflammatory component in both and is therefore also a non-discriminatory clinical sign.

TACO continues to be the leading cause of transfusion-related deaths in the UK and the number of cases reported to SHOT, the UK haemovigilance scheme continues to increase year on year (see Figure 19.4). The 2020 reporting year recorded 149 TACO cases which is the highest ever reported to SHOT.

Table 19.12 TACO classification criteria.

Required criteria	Acute or worsening respiratory compromise
	Evidence of acute or worsening pulmonary oedema on physical examination or chest x-ray or other non-invasive assessment of cardiac function
Additional criteria	Cardiovascular system changes not explained by the patient's underlying medical condition, e.g. tachycardia, hypertension, raised jugular venous pressure, cardiomegaly on chest x-ray, peripheral oedema
	Evidence of fluid overload, e.g. positive fluid balance, clinical improvement with diuresis
	Supportive result of a relevant biomarker, e.g. raised B-natriuretic peptide or N-terminal pro-brain natriuretic peptide

Source: Original data from Osselton et al. Lancet Haematology, 2019.

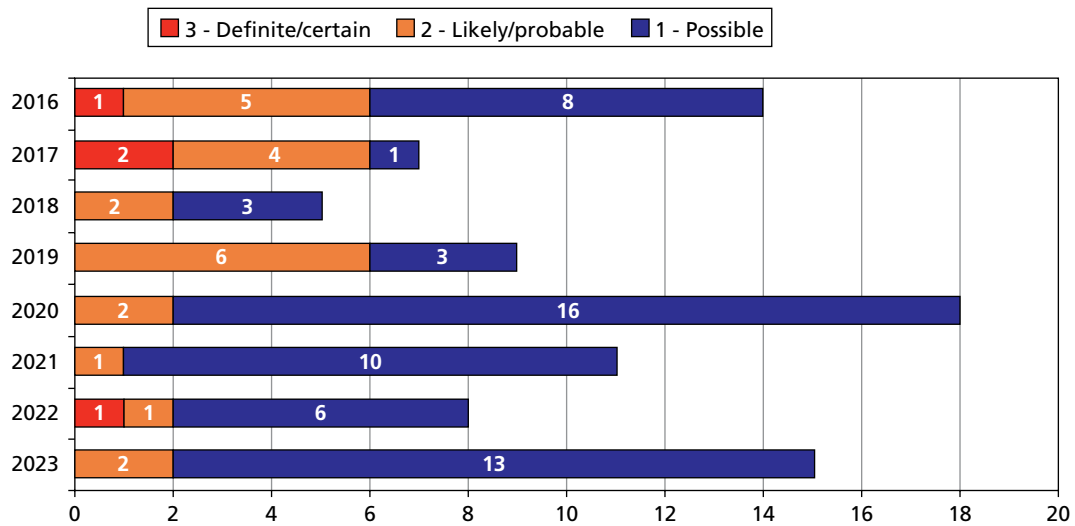

Figure 19.4 Deaths related to TACO reported to SHOT (with imputability) 2016–2023.

Furthermore, one-third of the TACO deaths reported to SHOT between 2016 and 2020 were deemed to be preventable, highlighting the importance of TACO risk assessment pretransfusion for all vulnerable patients.

The risk factors for TACO include age >70 years and comorbidities, including cardiac failure, renal impairment, severe anaemia and hypoalbuminaemia. Low-weight patients, such as the frail elderly and children, are at increased risk of receiving inappropriately high-volume and rapid blood transfusions, predisposing to TACO. Comprehensive pretransfusion assessment, with particular attention to the speed and volume of transfusion, fluid balance monitoring and use of diuretics, if indicated, may help identify at-risk patients and prompt mitigating measures to prevent this complication. SHOT data show that there is suboptimal use of pretransfusion TACO risk assessment and weight-adjusted red cell dosing is not sufficiently implemented.

Other adverse effects of transfusion

Haemosiderosis is a complication of repeated blood transfusions, seen more commonly as long-term blood transfusion therapy improves the survival of patients suffering from some chronic anaemias. It is most commonly seen in patients with thalassaemia, who commence transfusions in early childhood; increased iron absorption due to ineffective erythropoiesis compounds the iron overload in these patients. Transfusion-related iron overload can also be seen in people with other multi-transfused disorders such as sickle cell disease, other inherited anaemias, aplastic anaemia, myelodysplastic syndromes, other haematological malignancies and those who have undergone haemopoietic stem cell transplantation. Each unit of blood contains approximately 250 mg of iron, whereas the daily excretion rate is about 1 mg, and the body has no way of excreting the excess. Unless a patient is actively bleeding, hence losing iron, iron accumulation is inevitable.

For individuals without iron deficiency, it is generally accepted that transfusion of more than 15–20 units of RBCs (more than 10 units in smaller children) can cause clinically significant iron overload. However, some individuals with ineffective erythropoiesis who also have increased iron absorption may develop iron overload earlier. The correlation between the total volume of transfusions and clinical manifestations of iron overload is not exact, and clinical judgement is required in determining when to assess for iron overload. Optimal use of blood components and avoiding unnecessary transfusions are vital strategies to reduce the risk of iron overload. Chelation reduces morbidity in patients with transfusion dependency and iron overload, and it is routine practice in the management of patients with transfusion-dependent thalassaemia.

In addition to the more tangible adverse events of transfusion described above, there are increasing concerns about the impact of transfusion on overall patient outcome in relation to mortality and morbidity in critically ill patients, for example with potential impact on postoperative infection rates and length of hospital stay after surgery. This has led to more restrictive transfusion practices as described below. There is also an ongoing focus on the age of blood, with questions around the clinical impact of the age of blood on patient outcomes being tackled by randomised controlled studies.

Haemovigilance

Haemovigilance is defined by the International Haemovigilance Network as 'a set of surveillance procedures organised from the collection of blood and its components to the follow-up of its recipients, with the purpose of collecting and evaluating information on the undesirable and unexpected effects resulting from the therapeutic use of labile blood components and of preventing their occurrence or recurrence'.

The UK developed the first voluntary system in 1996, the SHOT – reporting is now professionally mandated and has ~100% participation from healthcare organisations across the UK. International haemovigilance data have resulted in changes in policy, products and practice, leading to improved blood donor safety, optimised product use and better clinical outcomes after transfusion.

SHOT is the UK's independent, professionally-led haemovigilance system, receiving reports of serious adverse transfusion reactions, errors and also near-miss events. There is a legal requirement to report serious adverse reactions and events to the MHRA under the Blood Safety and Quality Regulations 2005 in the UK. SHOT and MHRA now work closely together and have a joint reporting system.

Data collected show a reduction in the frequency of many of the most serious events, such as ABO-incompatible transfusions, transfusion-transmitted infections and TRALI. These trends follow changes in national policies on training and competency for staff involved in blood transfusion and a switch to male-only FFP. Overall mortality directly attributable to transfusion has decreased. However, errors related to transfusion remain the largest category of serious events, emphasising the significant potential for further improving safety. Fostering a strong and effective safety culture that is 'just and learning' is vital to ensure reductions in transfusion incidents and errors, thus directly improving patient safety.

SHOT also collects data on 'near-miss' events most of which are wrong blood-in-tube incidents highlighting the need for further improvements, particularly to ensure accurate patient identification. Whilst SHOT does not comprehensively collect data on fractionated plasma products, reports on events related to the inappropriate administration of anti-D Ig and prothrombin complex concentrates are collated with feedback on key learning points.

Appropriate use of blood and alternatives to allogeneic blood transfusion

In view of the inherent risks of blood transfusion and of the dependency of the blood supply on volunteer donors, blood and components should only be transfused when the benefits outweigh the risks. In addition to the clearly defined adverse events of transfusion, there are several reasons for aiming to reduce unnecessary allogeneic blood transfusions:

1 safety of the patient, by avoiding errors, as well as microbiological and immunological risks;
2 increasing difficulties in the recruitment of blood donors;
3 cost containment, with the ever-increasing demands by the regulatory agencies and technological advances;
4 high anxiety levels in patients, disproportionate to the real residual infectious risk of transfusion.

Patient blood management (PBM)

PBM is an evidence-based, multidisciplinary approach to optimising the care of patients who might need transfusion, considering alternatives, where feasible, and giving transfusion only when appropriate. PBM covers all aspects of decision-making in transfusion therapy, including initial patient evaluation with use of appropriate indications and transfusion triggers, minimising blood loss and optimisation of the patient's haemoglobin wherever possible. The application of the principles of PBM can reduce the need for transfusion and therefore health care costs whilst ensuring that limited donor blood supplies are available for the patients who need them most. In the surgical setting, the principles of PBM have been described as the 'three pillars', but it is important to note that the overall PBM strategy applies to all patients who might need transfusion, whether adults or children, elective or emergency, in any clinical setting, i.e. medical, surgical, obstetrics, intensive care, etc.

There should be clear guidelines for safe and appropriate use of blood components in all clinical specialities. These should be based on national and international data, with appropriately graded recommendations reflecting the quality of evidence available within the published literature. These should be complemented by regular multidisciplinary audits of transfusion practice, with feedback of results to all relevant staff and with re-audit where needed. The National Comparative Audit of Transfusion funded by NHS Blood and Transplant has active participation from hospitals across the country.

Although there has been a significant decrease in overall blood usage in the UK in the last couple of decades, several large regional and national audits of the use of red cells, platelets and plasma components continue to show ~15–20% inappropriate use, with ongoing variation in practice, highlighting the ongoing need to implement effective PBM.

Clinical decision to transfuse

The decision to transfuse requires a careful assessment of the patient's overall clinical status as well as their haematological laboratory data. Timely information should be made available to patients, ideally in written leaflets, about the indication for transfusion, the risks and benefits, and any alternatives available, in accordance with the principles of valid consent. The indication for transfusion and the patient's consent should be documented in the clinical records, along with the initial assessment including evaluation of the patient's TACO risk. Any special requirements, e.g. extended Rh- and K-matched blood for haemoglobinopathy patients or irradiated components for haemato-oncology patients should be considered when prescribing and requesting blood and components from the transfusion laboratory. Single-unit red cell transfusions are recommended where possible, especially in non-bleeding adult patients, and further units should not be prescribed without further clinical assessment and monitoring of the patient's haemoglobin. Computer-assisted clinical decision-making may help with appropriate transfusion therapy, but such systems need significant resources and are still not widely available.

Strategies to minimise transfusion of blood and components

Early preoperative identification of anaemia, as part of a general preoperative assessment, (see Table 19.13), may be followed by appropriate management with haematinics, and can reduce the

Table 19.13 Preoperative assessment of patients.

- Take a full history and examination, including previous surgical episodes and bleeding history, transfusion and obstetric history
- Arrange full blood count (FBC), group and antibody screen, routine chemistry, consider coagulation screen (not routinely needed unless specific clinical indication) and tube for haematinics assessment (ferritin level for iron stores, vitamin B_{12} and folate), which can be put on hold pending FBC results
- Consider use of tranexamic acid to prevent surgical blood loss
- Make cell salvage available if there is a likelihood of significant blood loss requiring transfusion and a 'clean' surgical procedure
- If iron deficiency give oral iron; use intravenous iron if intolerant or unresponsive to oral iron or if short timeframe prior to surgery (<2 weeks)
- Prescribe folic acid supplement or B12 if evidence of deficiency
- If patient on antiplatelet therapy or oral anticoagulant drugs then assess and implement appropriate management plan to minimise risk of bleeding in the perioperative period

need for transfusion. Appropriate management of anticoagulants and antiplatelets agents in this setting may also reduce the need for subsequent transfusion. Iron deficiency anaemia is also common in the obstetric setting, with often inadequate systems in place for timely intervention; oral or intravenous iron, as required, should be prescribed. Active management of anaemia is also indicated in the medical setting, with targeted management of true or functional iron deficiency, other haematinic deficiency (i.e. B12 and folate) or anaemia of chronic disease. The volume of blood samples taken at phlebotomy should be minimised since this can exacerbate anaemia, particularly in premature babies and the intensive care setting.

Surgical and anaesthetic techniques, and the use of fibrin glues and sealants, provide additional scope for the avoidance of donor transfusion in the context of surgery. Intraoperative cell salvage should be used where feasible but preoperative autologous blood deposit is no longer favoured, as evidence of efficacy is patchy and contradictory. High-quality evidence now supports the use of tranexamic acid in trauma and major obstetric haemorrhage but not in acute gastrointestinal bleeding. Patients with inherited bleeding disorders need careful planning and management plans discussed with appropriate expert input around options e.g. desmopressin (DDAVP) in patients with mild haemophilia. Erythropoietin can help reduce red cell requirements but is expensive and therefore of limited application. Point-of-care testing may aid decision-making around use of plasma components and platelets but validated protocols supported by training and appropriate quality control are essential where these techniques are used.

Red cell transfusion triggers

The seminal Transfusion Requirements in Critical Care (TRICC) study demonstrated no mortality benefit for patients with a higher transfusion threshold when compared to a restrictive haemoglobin trigger. The results of the TRICC study are strongly supported by further studies in high-risk patients needing hip surgery, cardiac surgery and acute upper gastrointestinal bleeding. This evidence from prospective, randomised, controlled studies and meta-analyses demonstrates that a restrictive transfusion strategy results in equivalent or possibly better clinical outcomes than more liberal transfusion and should inform clinical decision-making. Accordingly, a threshold for red cell transfusion of 70–80 g/L haemoglobin is appropriate in the majority of patients. A higher threshold of 80–90 g/L may be indicated in patients with acute coronary syndromes, pending further studies.

Platelet transfusions

Platelet transfusions are indicated for the prevention and treatment of haemorrhage in patients with thrombocytopenia or platelet function defects. The largest group of patients, receiving up to 67% of all platelet concentrates, are haematology patients. Most of these platelet transfusions are prophylactic. The decision to transfuse platelets should be based on clinical assessment, taking into account clinical risk factors for bleeding and the extent and site of bleeding, together with the platelet count.

There has been increasing research to try and support clinical practice in this area. Randomised, controlled studies have attempted to tackle key questions around decision-making on prophylactic platelet transfusions in haemato-oncology patients. In such patients, the platelet count threshold for prophylaxis is 10×10^9/L, but a higher threshold may be indicated, depending on additional patient risk factors e.g. sepsis. The dose of prophylactic platelets has been shown to have no effect on the incidence of bleeding – accordingly, only single doses ($2.4–3 \times 10^{11}$) of platelets should be administered prophylactically, with little justification for routine use of higher, or 'double', doses of platelets. More recent randomised controlled studies highlight the need for caution in certain clinical settings. Amongst preterm infants with severe thrombocytopenia, those randomly assigned to receive platelet transfusions at a platelet-count threshold of 50×10^9/L had a significantly higher rate of death or major bleeding than those treated at a platelet-count threshold of 25×10^9/L (PlaNeT 2 MATISSE study). Platelet transfusion seems inferior to standard care in patients with acute intracranial haemorrhage associated with antiplatelet therapy (PATCH study).

There is a lack of evidence for platelet thresholds, indicating the need for transfusion, in other clinical settings and, in particular, prior to procedures or surgery, with the use of empirical guidance to support practice e.g. 50×10^9/L for minor surgery and 80×10^9/L for major surgery.

Platelet transfusion response and refractoriness

The efficacy of platelet transfusions can be assessed by monitoring clinical response and by checking the platelet count increment. In patients requiring routine prophylactic platelet transfusions, a repeated failure to obtain a satisfactory response to two consecutive ABO-matched, relatively fresh platelet transfusions signifies refractoriness to platelet transfusions. Formulae to derive platelet recovery or corrected count increment are of limited value in the clinical setting and simpler, more pragmatic indicators include a failure of the post-transfusion (10 minutes to an hour) platelet increment to exceed the pretransfusion count or a rise of less than 10×10^9/L after 24 hours post-transfusion.

Refractoriness to platelet transfusions is commonly due to non-immunological causes associated with increased platelet consumption or losses such as infections, antimicrobials (amphotericin B and fluoroquinolones), splenomegaly/hypersplenism, DIC and major haemorrhage. Immunological refractoriness to platelet transfusions is due mainly to antibodies against antigens on platelets (HLA, HPA, ABO) and, more rarely, to platelet autoantibodies and drug-dependent antibodies or immune complexes. The most common cause of

immunological refractoriness is the presence of HLA antibodies in the recipient. Although HLA alloimmunisation is less common following universal leucodepletion of blood components, it is still a major problem in haemato-oncology patients needing prophylactic platelet transfusions.

The refractory patient should be assessed for the presence of non-immunological causes and if these have been excluded, and ABO-matched platelets have caused no increment, screening for HLA antibodies should be undertaken. If these are present, then platelet transfusions, collected by apheresis, from donors matched as closely as possible with the patient's HLA-A and HLA-B antigens should be given. The blood services in the UK have a large panel of HLA-typed donors, but this is not so in most countries, where the only alternative is to give cross-match-compatible platelets from the available stock. Immediate (10–60 minutes) and 24-hour post-transfusion platelet increments should be measured for evidence of effectiveness of therapy. If there is no response, then screening for HPA and other less common antibodies should be considered after advice from a transfusion medicine specialist. HLA-matched platelet preparations should be irradiated to prevent TA-GvHD. There has been some research in the use of epitope-matched platelets to support HLA-alloimmunised patients but these are not available for clinical use.

Use of fresh-frozen plasma (FFP) and cryoprecipitate

There is a relative lack of good evidence to support the clinical use of FFP and therefore transfusions are given empirically in many situations. FFP is indicated in patients with acute DIC in the presence of bleeding and abnormal coagulation results. The indication for FFP use in major haemorrhages is described below. FFP can also be used to treat inherited single coagulation factor deficiencies if the appropriate factor concentrates are not available. FFP should not be used for reversal of oral anticoagulation where the use of prothrombin complex concentrate is indicated. The coagulopathy of liver disease is also complex, with many studies showing the lack of evidence of clinical benefit of prophylactic FFP. Previous audits have shown that a large proportion of the FFP used in the UK and in many other countries is misused, exposing patients to unnecessary risks.

Cryoprecipitate is indicated for fibrinogen replacement largely in the major haemorrhage setting (see below). Cryoprecipitate must not be used for replacement of coagulation factors in inherited conditions such as haemophilia or von Willebrand disease, since specific factor concentrates are available.

Major haemorrhage

Major haemorrhage can occur in many settings, e.g. trauma, obstetric, GI bleeds, with significant challenges in management. Red cell transfusion is usually required when 30–40% of blood volume is lost (1500 mL in 70 kg male); more than 40% blood volume loss (1500–2000 mL) is life-threatening and requires immediate transfusion. Red cells are necessary for their oxygen-carrying capacity and also because they contribute to improved haemostasis through a rheological effect leading to axial flow and therefore margination of platelets.

Metabolic changes in stored blood include low pH, hypocalcaemia and hyperkalaemia. The reduced oxygen-carrying capacity of stored blood becomes significant only after 21 days' storage (for CPD-AI blood) and is due to low 2,3-DPG levels. Although, theoretically, excess citrate in transfused blood could cause toxicity, its metabolism in the liver is usually rapid. In practice, the only situations when citrate toxicity is a real problem is with extremely rapid transfusion (one unit every 5 minutes), or in infants, especially if premature, having exchange transfusion with blood stored in citrate for longer than 5 days. Hypocalcaemia and hyperkalaemia are usually transient and rapidly corrected once the transfused blood is circulating. Acidosis is not generally significant, as citrate metabolism leads to an alkalosis. However, if a patient is severely shocked and under-transfused, acidosis may be a clinical problem. All these changes due to stored blood are exacerbated by hypothermia. Cardiac irregularities, in particular ventricular fibrillation, may result from transfusion of large quantities of cold blood. The optimal functioning of coagulation factors and of platelets is also temperature dependent and effectiveness is reduced by hypothermia. Thus, the use of a blood warmer and keeping the patient warm are important measures in the management of patients with major haemorrhage.

Approximately one-quarter of patients bleeding with severe trauma present with clotting abnormalities, indicating that the coagulopathy is not merely dilutional related to infusion of fluids or red cells. Indeed, coagulopathy associated with massive haemorrhage is likely to be multifactorial with contributory factors, including activation of fibrinolysis and consumptive coagulopathy, exacerbated by hypothermia and hypocalcaemia. The use of anticoagulant/antiplatelet drugs prior to surgery may further contribute to bleeding post-surgery together with the heparin needed for bypass for cardiac surgery.

The practical management of major haemorrhage in any setting needs a coordinated, multidisciplinary approach. This should incorporate significant advances in techniques for resuscitation as well as surgical, radiological and endoscopy interventions to control bleeding.

Baseline blood samples should be taken for FBC, chemistry, coagulation screen and group and screen. In patients with severe haemorrhage, the initial use of group O red cells is indicated before the blood group is available; RhD-negative units should be given to females of childbearing age. A switch to group-specific blood should be made as soon as possible.

The CRASH-2 study has demonstrated the benefits of early use of antifibrinolytics in trauma and therefore tranexamic acid should be incorporated into trauma protocols. Tranexamic acid

is also of benefit in major obstetric haemorrhage as shown by the WOMAN trial. However the HALT- IT study showed that it should not be routinely used for patients with severe gastrointestinal bleeding.

There has been extensive interest in the effects of early and/or high-dose empirical plasma therapy on clinical outcomes in traumatic haemorrhage. Many observational studies concluded that higher ratios of FFP:RBC were associated with reduced mortality (confounding by survivor bias is a concern). Many UK centres use empiric FFP:RBC ratios of 1:1 for traumatic bleeding. Data are unclear regarding the role of early administration of platelets; an empirical approach of administration if the count falls below 75×10^9/L is often used in major haemorrhage.

Either cryoprecipitate or fibrinogen concentrate can be used for replacement depending on the product availability and licensing within the country. A target of a fibrinogen level of 1.5 g/L is used in traumatic haemorrhage or 2.0 g/L in obstetric haemorrhage.

There has been a resurgence of interest in the use of whole blood in the management of traumatic major haemorrhage as opposed to individual component therapy as a more balanced approach to replacement. In the UK, researchers are studying the pre-hospital clinical use of a 'Red Cell and Plasma' product whilst further exploring development of a suitable platelet-rich, leucodepleted 'whole blood' product.

Haemolytic disease of the foetus and newborn (HDFN)

HDFN is a condition in which the lifespan of the foetal/neonatal red cells is shortened due to maternal alloantibodies against red cell antigens inherited from the father. Maternal IgG can cross the placenta, and thus IgG1 and IgG3 red cell alloantibodies can gain access to the foetus. If the foetal red cells contain the corresponding antigen, then binding of antibody to red cells will occur. When the antibody is of clinical significance (e.g. anti-D, -c, -K), and of sufficient potency, the coated cells will be prematurely removed by the foetal mononuclear phagocytic system. The effects on the foetus/newborn infant may vary according to the characteristics of the maternal alloantibody. The blood film of a foetus affected by HDFN shows polychromasia and increased numbers of nucleated red cells (Figure 19.5). In most cases (except a few due to ABO antibodies), the DAT (Coombs' test) on the infant's cells is positive owing to IgG coating.

The antibodies giving rise to HDFN most commonly belong to the Rh or ABO blood group systems. The morbidity of Rh HDFN is explained by the great immunogenicity of the RhD antigen; HDFN due to anti-c is also important and its incidence comes second amongst the cases of severe HDFN, closely followed by anti-K. (The disease caused by anti-K is more properly called alloimmune anaemia of the foetus and newborn as it is due to direct inhibition of erythropoiesis by the antibody and haemolysis is not a feature.) Antibodies against antigens in almost all the blood group systems (e.g. Duffy, Kidd), and against the so-called 'public' and 'private' antigens, have also been occasionally responsible for HDFN. However, IgM cannot cross the placenta and Lewis and P_1 antibodies, which occur frequently during pregnancy, are usually IgM and do not lead to HDFN. Furthermore, the Lewis antigens are not red cell antigens per se and are not fully developed at birth.

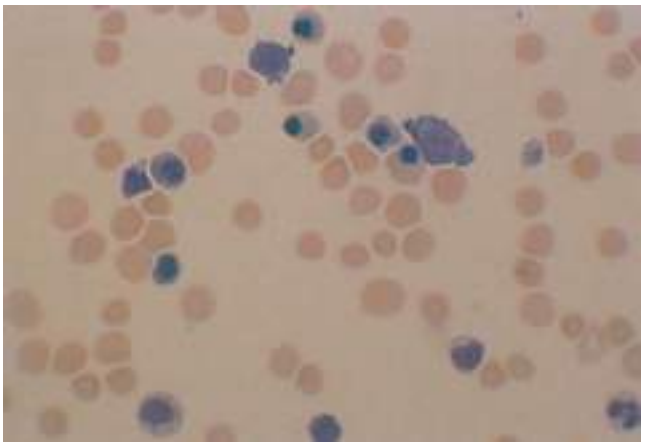

Figure 19.5 Blood film of a foetus affected by HDFN showing polychromasia and increased numbers of normoblasts.

Some antibodies (particularly anti-D, -K and -c) are associated with significant foetal and neonatal risks, such as anaemia requiring intrauterine or neonatal transfusion, jaundice or perinatal loss due to hydrops foetalis or kernicterus. There are many antibodies that are unlikely to significantly affect the foetus, but can cause neonatal anaemia and hyperbilirubinaemia), whilst others may cause problems for the screening and timely provision of appropriate blood for the mother or foetus/neonate. ABO antibodies can also cause mild to moderate anaemia and jaundice in the neonate and very occasionally in the foetus. In particular, IgG anti-A, B, anti-A and anti-B in group O mothers can cross the placenta and frequently cause HDFN in group A or B offspring.

The blood group and antibody status of the mother should be tested at booking and at 28 weeks of gestation to identify the ABO group and RhD status and to detect clinically significant red cell antibodies (see Figure 19.6). If red cell antibodies are detected in the booking sample, further testing of maternal blood should be undertaken to determine the specificity and level of antibody or antibodies, and to assess the likelihood of them causing HDFN (Figure 19.7). The levels of anti-D and anti-c are quantified using automated analysers, whereas the titre of all other antibodies is determined by doubling dilution. Once detected, anti-D, anti-c and anti-K levels should be measured every 4 weeks up to 28 weeks' gestation and then every 2 weeks, until delivery. All other antibodies should be retested at 28 weeks gestation. Referral to a foetal

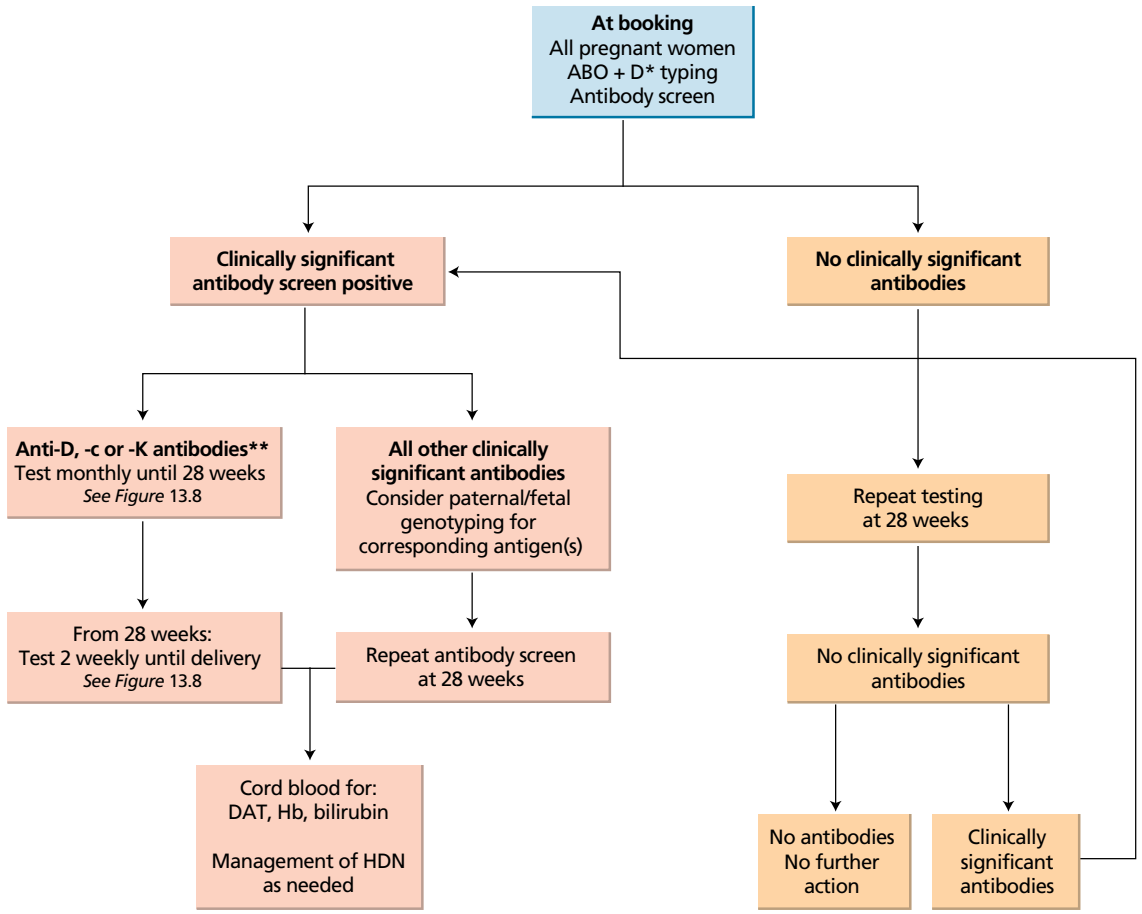

Figure 19.6 Blood group and antibody testing in pregnancy. If D-negative mother with no immune anti-D, advise anti-D Ig prophylaxis for any PSEs in pregnancy and give routine antenatal anti-D Ig prophylaxis (RAADP), either as a single dose or as two doses (see RCOG anti-D guidelines); after delivery check cord sample for D type and maternal sample for fetomaternal haemorrhage (e.g. Kleihauer) testing to check if further anti-D Ig needed, in addition to the standard dose, which should be given in the first instance after delivery. DAT, direct antiglobulin test; Hb, haemoglobin; RAADP, routine antenatal anti-D Ig prophylaxis. (Source: RCOG Greentop guidelines 65, 2014. Reproduced with permission of The Royal College of Obstetricians and Gynaecologists). **Pregnancies with immune anti-D, -K or -c are at particular risk of severe foetal HDFN, so further early assessment and referral to foetal medicine specialist is indicated.

medicine specialist should occur when there are rising antibody levels, but referral should also take place if there is a history of previous HDFN or of pregnancy with unexplained severe neonatal jaundice or anaemia requiring transfusion or exchange transfusion.

An anti-D level of >4 IU mL but <15 IU/mL correlates with a moderate risk of HDFN and an anti-D level of >15 IU/mL can cause severe HDFN. An anti-c level of >7.5 IU/mL but <20 IU/mL correlates with a moderate risk of HDFN, whereas a level of >20 IU/mL correlates with a high risk of HDFN. For anti-K, severe foetal anaemia can occur even with low titres, so early referral to a foetal medicine specialist is advisable.

Where clinically significant maternal red cell antibodies are detected, the father's sample should also be tested for paternal phenotype to determine the risk of the foetus carrying the relevant antigen. If the partner is a heterozygote or not available, free foetal DNA (ffDNA) extracted from maternal plasma can be tested for RhD and Rhc genotype from 16 weeks onward and for K from 20 weeks (see Chapter 18).

If the foetus is antigen-positive, then the pregnancy must be assessed and monitored for the development of HDFN by a foetal medicine specialist. Non-invasive techniques have largely replaced invasive testing, in particular with Doppler flow velocity; the use of middle cerebral artery (MCA) peak systolic velocity is a highly sensitive method for detecting foetal anaemia (see Figure 19.8). Ultrasound monitoring may help in detecting features suggesting severe foetal anaemia including polyhydramnios, skin oedema and cardiomegaly. The MCA peak systolic velocity related to gestation helps guide the need for further intervention including foetal blood sampling (see Figures 19.8 and 19.9) and IUTs. The specifications for blood used for IUT are shown in Table 19.7.

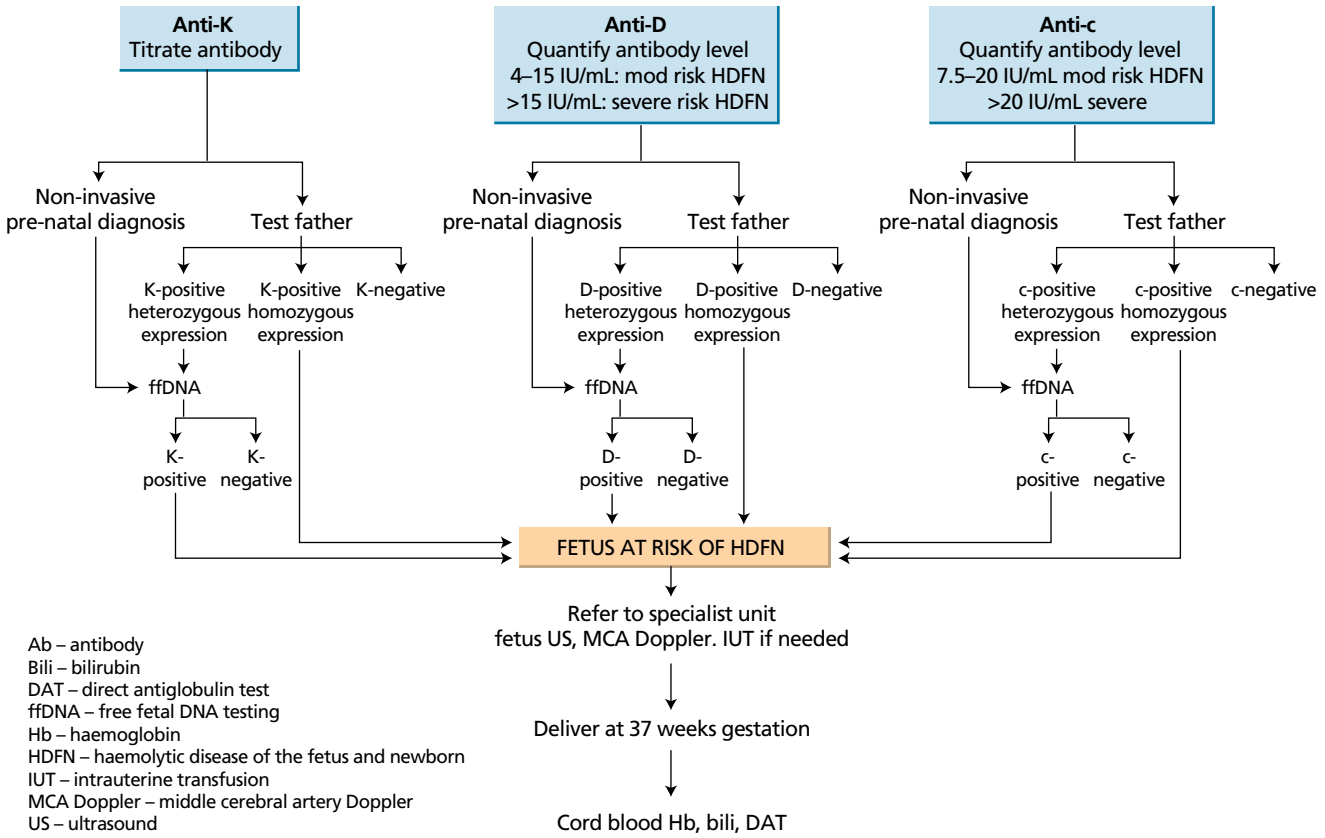

Figure 19.7 Management algorithm for pregnancies complicated with anti-D, anti-K or anti-c alloimmunisation from RCOG Greentop guideline 2014. (Source: RCOG Greentop guidelines 65, 2014. Reproduced with permission of The Royal College of Obstetricians and Gynaecologists)

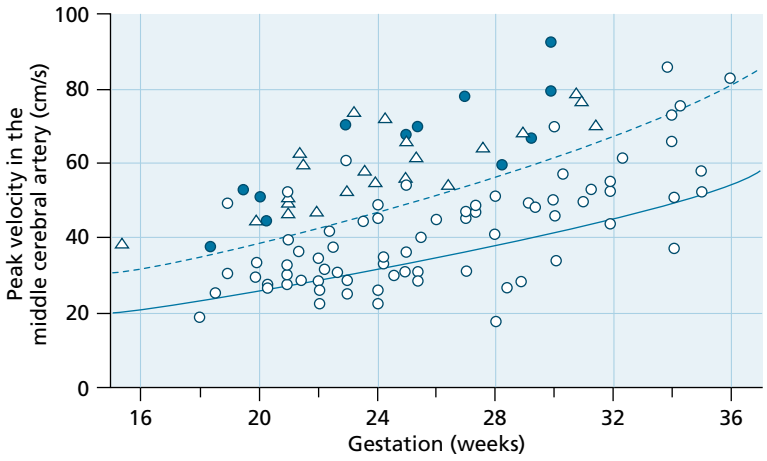

Figure 19.8 Middle cerebral artery Doppler. Peak velocity of systolic blood flow in the middle cerebral artery in 111 foetuses at risk for anaemia due to maternal red cell alloimmunisation. Open circles indicate foetuses with either no anaemia or mild anaemia (≥0.65 multiples of the median Hb concentration). Triangles indicate foetuses with moderate or severe anaemia (<0.65 multiples of the median Hb concentration). Solid circles indicate the foetuses with hydrops. Solid curve indicates the median peak systolic velocity in the middle cerebral artery and the dotted curve indicates 1.5 multiples of the median. (Source: Professor Charles Rodeck. Reproduced with permission)

Where the mother is known to have immune red cell antibodies, at delivery, cord blood should be taken for ABO and D typing and also for DAT, Hb and bilirubin levels. A positive DAT indicates that the infant's red cells are coated with antibodies, but in itself cannot predict the severity of haemolysis, if any. Notably, the DAT may be negative in ABO HDFN. It is therefore essential to also determine the Hb and bilirubin level to ascertain the degree of anaemia and haemolysis at birth, as this helps guide management, either with phototherapy or transfusion. The specifications for blood for exchange transfusion are shown in Table 19.5. Some infants may have anaemia persisting for a few weeks following birth. Whilst this may be due to passively acquired maternal antibodies causing continued haemolysis, it may also be due to suppression of erythropoiesis with extremely

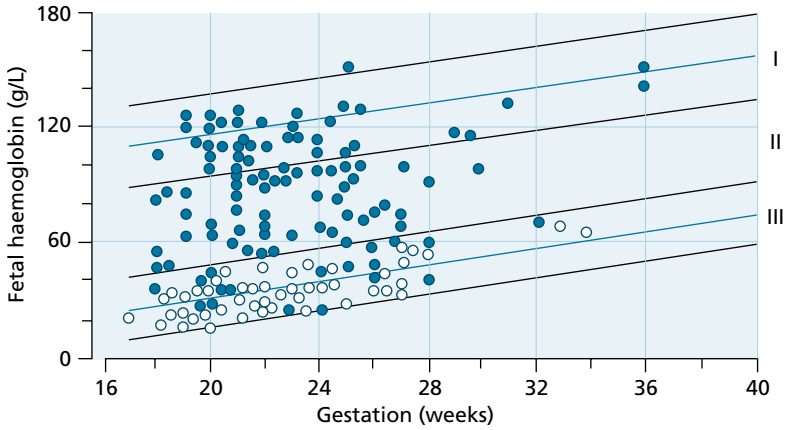

Figure 19.9 Foetal haemoglobin (Hb) concentration of 48 hydropic (open circles) and 106 non-hydropic (closed circles) foetuses from red cell isoimmunised pregnancies at the time of first blood sampling. Values are plotted on the reference range of foetal Hb for gestation. The individual 95% confidence intervals of the normal Hb for gestation define zone I and the individual 95% confidence intervals of the Hb for gestation of the hydropic foetuses define zone III. Zone II indicates moderate anaemia. (Source: Professor Charles Rodeck. Reproduced with permission)

low reticulocytes in the affected infants and further top-up transfusions may be needed.

Prevention of RhD HDFN

The introduction of prophylaxis with anti-D immunoglobulin (anti-D Ig) for D-negative mothers has greatly reduced the risk of HDFN, a previously significant cause of morbidity and mortality. Prior to the availability of anti-D prophylaxis, the first ABO-compatible, RhD-positive offspring resulted in the primary immunisation of about 17% of D-negative women. The rate of alloimmunisation fell considerably after administration of anti-D Ig postpartum and following other potentially sensitising events (PSEs) during pregnancy, as listed in Table 19.14. It was, however, recognised that 'silent' transplacental bleeds could still occur in the later stages of pregnancy and the introduction of routine antenatal anti-D prophylaxis (RAADP) further reduced the sensitisation rate to very low levels, ranging from 0.17 to 0.28%. Associated with this reduction in sensitisation is a reduction in mortality due to HDFN, from 46/100,000 births to 1.6/100,000 births.

Following sensitising events, anti-D Ig should be administered as soon as possible and ideally within 72 hours of the event. If, exceptionally, this deadline has not been met, some protection may be offered if anti-D Ig is given up to 10 days after the sensitising event. In pregnancies less than 12 weeks' gestation, anti-D prophylaxis is only indicated following ectopic pregnancy and therapeutic termination of pregnancy, but may also be indicated in some cases of uterine bleeding, where this is repeated, heavy or associated with abdominal pain. For events between 12 and 20 weeks' gestation, a minimum dose of 250 IU should be given. After 20 weeks' gestation, a minimum anti-D Ig dose of 500 IU is indicated and testing for fetomaternal haemorrhage (FMH) should be performed to determine if additional anti-D Ig is needed.

The National Institute for Health and Care Excellence (NICE) has recommended that D-negative pregnant women who do not have immune anti-D, should be offered RAADP during the third trimester of pregnancy. This can be with a single-dose regimen at 28 weeks, or two-dose regimen given at 28 and 34 weeks; practice has now shifted mainly towards a single-dose regimen due to ease of administration and availability of doses. It is important that the 28-week sample for blood group and antibody screen is taken prior to the first routine prophylactic anti-D Ig injection. RAADP should be regarded as a separate entity and administered regardless of, and in addition to, any anti-D Ig that may have been given for a sensitising event.

Following birth, the cord blood should be tested and if the baby is D-positive, all D-negative, previously non-sensitised, women should be offered at least 500 IU anti-D Ig within 72 hours following delivery. Maternal samples should be tested for FMH and additional dose(s) given as guided by FMH tests (see below). If there is an intrauterine death (IUD) and no sample can be obtained from the baby, an appropriate dose of prophylactic anti-D Ig should be administered. Anti-D prophylaxis is also indicated if intraoperative cell salvage is used during caesarean section in D-negative women, where the cord blood group is confirmed as D-positive (or unknown) with estimation of FMH on a maternal sample taken 30–45 minutes after reinfusion to check if additional doses of anti-D Ig are needed.

Table 19.14 Potentially sensitising events in pregnancy.

- Amniocentesis, chorionic villus biopsy and cordocentesis
- Antepartum haemorrhage/PV bleeding in pregnancy
- External cephalic version
- Fall or abdominal trauma (sharp/blunt, open/closed)
- Ectopic pregnancy
- Evacuation of molar pregnancy
- Intrauterine death and stillbirth
- *In-utero* therapeutic interventions (transfusion, surgery, insertion of shunts, laser)
- Miscarriage, threatened miscarriage
- Therapeutic termination of pregnancy
- Delivery – normal, instrumental or caesarean section
- Intraoperative cell salvage

Source: From BCSH guidelines 2014.

A dose of 500 IU intramuscularly (IM) is considered sufficient to treat an FMH of up to 4 mL foetal red cells. Where it is necessary to give additional doses of anti-D Ig, as guided by tests for FMH, the dose calculation is traditionally based on 125 IU anti-D Ig/mL foetal red cells for IM administration. However, the manufacturer's guidance should be referred to, depending on which product is used. In cases of large FMH, and particularly if FMH is in excess of 100 mL, a suitable preparation of intravenous anti-D Ig should be considered. Good communication between the antenatal teams and the transfusion laboratory is essential, with clear documentation of issues and administration of anti-D immunoglobulin. In the UK, the SHOT haemovigilance scheme continues to report on many errors with delayed or missed administration of anti-D prophylaxis.

Foetal RhD Screening

Several countries including the UK have now introduced a non-invasive pre-natal screening test using ffDNA in maternal plasma to determine foetal D blood group antigen status. This is able to predict the foetal D status from 11+2 weeks gestation with >99.9% negative predictive value, meaning the approximately 40% of D-negative women carrying a D-negative foetus would no longer require unnecessary anti-D injections as part of RAADP, antenatal sensitising event prophylaxis or post-partum prophylaxis.

A NICE review of the technology in 2016 recommended that it is cost-effective due to the cost-savings in reduced attendances to receive anti-D, reduced anti-D Ig usage and reduced lab testing for FMH estimation. Critically, it avoids unnecessary exposure of women to a blood product.

Testing for fetomaternal haemorrhage (FMH)

Commonly used techniques for quantification of FMH include the acid elution method, a modification of the Kleihauer–Betke test and flow cytometry.

The principle of the Kleihauer test relies on the different properties of foetal haemoglobin (HbF) and adult haemoglobin (HbA), whereby HbF is more resistant than HbA to both acid elution and alkaline denaturation. When a blood film from the maternal sample is fixed and immersed in an acid buffer solution, HbA is denatured and eluted, leaving red cell ghosts. Foetal red cells containing HbF that are resistant to elution stand out after staining in a background of maternal ghost cells. The relative percentages can then be counted using light microscopy to calculate an estimated volume of FMH. It is essential to have positive and negative controls. This is a manual test with a high coefficient of variation. Accordingly, current BSH guidelines recommend that more accurate testing with flow cytometry should be undertaken if the estimated FMH volume is >2 mL.

Flow cytometry is used to quantify the population of RhD-positive foetal cells in the RhD-negative maternal sample, using a fluorochrome-conjugated IgG monoclonal anti-D to label the D-positive cells.

A maternal sample should be taken for FMH testing after any sensitising event occurring after 20 weeks' gestation. When the sensitising event is before 20 weeks, the foetal blood volume is considered insufficient to exceed that covered by the minimum anti-D Ig dose, as recommended above. FMH testing should also then be undertaken after delivery, following which a standard dose of anti-D should be given after the baby is confirmed to be D-positive on cord blood testing. The result of the FMH testing is then used to decide if additional doses of anti-D Ig are needed. If additional doses of anti-D Ig are indeed needed, then follow-up FMH testing is indicated to confirm clearance of D-positive cells from the maternal circulation. Repeat FMH testing should be undertaken after 48 hours if anti-D is given intravenously or 72 hours after an intramuscular dose.

Rh D-positive platelet transfusions

Whenever possible, D-negative platelets should be transfused to D-negative girls or women of childbearing potential, but on occasions, depending on availability and clinical need, it may be necessary to transfuse D-positive platelets. In these circumstances, prophylaxis against possible Rh alloimmunisation by red cells contaminating the platelet product may be needed. In the UK, a dose of 250 IU anti-D Ig is considered sufficient to cover up to five adult therapeutic doses of D-positive platelets given within a 6-week period. In severely thrombocytopenic patients with platelet counts of $\leq 30 \times 10^9$/L, anti-D Ig should be given subcutaneously, or if an appropriate preparation is available, intravenously, to avoid the risk of intramuscular bleeding following IM injection.

Inadvertent transfusion of RhD-positive blood to RhD-negative women of childbearing potential

When less than one unit of red cells has been transfused, the appropriate dose of anti-D Ig may be given (see above). If more than 15 mL have been transfused, the larger anti-D immunoglobulin preparations (1500 or 2500 IU), if available, should be considered with intravenous anti-D immunoglobulin being preferable, achieving adequate plasma levels immediately. The quantitation of D-positive red cells should be performed by flow cytometry after 48 hours if an intravenous dose of anti-D has been given, or 72 hours if an intramuscular dose has been given and further anti-D Ig given until there are no detectable D-positive red cells in circulation.

When more than one unit of D-positive blood has been transfused, a red cell exchange transfusion should be considered to reduce the load of D-positive red cells in the circulation and the dose of anti-D Ig required to suppress immunisation. A single blood-volume red cell exchange transfusion will achieve a 65–70% reduction in D-positive red cells; a double-volume exchange will achieve an 85–90% reduction. Shortly after the exchange transfusion, the residual volume of D-positive red

cells should be estimated using flow cytometry to guide anti-D Ig dosage needed.

Advice should be sought from a transfusion medicine specialist. The patient should be counselled regarding the implications of both non-intervention (for future pregnancies) and of treatment, including any hazards from receiving donated blood, the exchange procedure itself and of larger doses of anti-D Ig, including intravenous anti-D. Passive anti-D Ig given in large doses may remain detectable and tests for immune anti-D may be inconclusive for several months.

Foetal and neonatal alloimmune thrombocytopenia (FNAIT)

This is a condition in which the platelets of the foetus and newborn are destroyed by maternal platelet-specific alloantibodies against platelet antigens inherited from the father (see also Chapter 54). It is analogous to HDFN, but for platelets. Although more than 15 HPA systems have been described, most cases are due to anti-HPA-1a in alloimmunised HPA-1b mothers. The difference with FNAIT is that the offspring of the first pregnancy can be affected and that the potency of the platelet antibodies is often not correlated with the severity of the foetal or neonatal thrombocytopenia. The most serious complication is intracranial haemorrhage, which may lead to death or severe neurological sequelae. Pending confirmatory testing, transfusion of HPA-1a/5b-negative platelets is usually indicated (depending on neonatal clinical situation and platelet count). Management in the foetus/pregnant woman requires specialist foetal medicine care and usually involves intravenous immunoglobulin. Specialist transfusion medicine advice should also be sought.

Infected blood inquiry

This was a public statutory inquiry examining the circumstances in which men, women and children treated by National Health Services in the United Kingdom were given infected blood and infected blood products, in particular since 1970. The report was released in 2024 with key lessons and recommendations for all health care professionals but critical for those involved in transfusion practice. The risk of acquiring infection from transfusion is now extremely low but that risk can never be removed completely. The Serious Hazards of Transfusion (SHOT) UK Haemovigilance scheme continues to highlight other adverse events related to transfusion. An important ongoing key message is that blood transfusion must only be given where essential and patients must be fully informed about the risks, benefits and alternatives available.

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PART III

Lymphoid disorders

CHAPTER 20

The classification of lymphomas

20

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Introduction

The classification of lymphoid neoplasms is based on two major principles: stratification of the neoplasms according to their cell lineage and the definition of non-overlapping distinct diseases that are clinically relevant. The identification of these diseases is based on a combination of morphology, immunophenotype, genetic and molecular features and clinical manifestations. The 2001 WHO classification of lymphoid neoplasms was based on the Revised European–American Classification of Lymphoid Neoplasms (REAL) published by the International Lymphoma Study Group (ILSG) in 1994. The validation of this proposal in a large series of tumours and the publication of the 2001 third edition of the WHO classification developed by the Society of Haematopathology (SH) and European Association for Haematopathology (EAHP) under the invitation of the International Agency for Cancer Research (IARC), the WHO agency in charge of the publication of the classification of tumours. This classification was based on a broad consensus of pathologists with clinicians and scientists in an ad hoc Clinical Advisory Committee (CAC). This ended a long history of controversy between different classification schemes that used different terminologies and concepts amongst pathologists and clinicians in different parts of the world. The subsequent 4th edition of the WHO classification in 2008 and its update in 2017 followed the same process of broad international effort promoted by the EAHP and SH, invited by the IARC, together with the CAC of pathologists, clinicians, geneticists and molecular scientists that integrated criteria developed by different working groups, refined definitions of well-established diseases, incorporated new entities and developed new concepts and ideas related to the biology of lymphomas. Unfortunately, in 2021 the IARC rejected the previous collaboration of the EAHP and SH. These societies decided to follow the same process of previous classifications with the nomination of different working groups of pathologists, clinicians and scientists who discussed the required changes in different groups and reached consensus for the update of the classification in a new CAC meeting establishing the International Consensus Classification (ICC) (Table 20.1a). The IARC nominated a group of experts to develop the 5th edition of the classification (WHO-HAEM5) (Table 20.1b). This chapter revises the most common lymphoid neoplasms with emphasis in the defining criteria, elements of the diagnosis and main clinical implications.

Mature B-cell neoplasms

This is a heterogeneous group of neoplasias that in most cases are related to different steps of the normal B-cell differentiation process. The combination of morphological, phenotypic and molecular features is essential for the diagnosis of the different entities (Table 20.2).

Chronic lymphocytic leukaemia/small lymphocytic lymphoma

Chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) is a neoplasm of mature small B-cell lymphocytes that commonly express CD5 and CD23 and have dim expression of surface IgM/IgD, and CD20 (Table 20.2). The

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 20.1a International consensus classification of mature lymphoid neoplasms.

Mature B-cell neoplasms
Chronic lymphocytic leukaemia/small lymphocytic lymphoma
Monoclonal B-cell lymphocytosis
  Chronic lymphocytic leukaemia type
  Non-chronic lymphocytic leukaemia type
B-cell prolymphocytic leukaemia
Splenic marginal zone lymphoma
Hairy cell leukaemia
Splenic B-cell lymphoma/leuakemia, unclassifiable
  Splenic diffuse red pulp small B-cell lymphoma
  Hairy cell leukaemia-variant
Lymphoplasmacytic lymphoma
Waldenström macroglobulinaemia
Immunoglobulin M (IgM) monoclonal gammopathy of undetermined significance (MGUS)
  IgM MGUS, plasma cell type*
  IgM MGUS, not otherwise specified (NOS)*
Primary cold agglutinin disease*
Heavy chain diseases
  Mu heavy chain disease
  Gamma heavy chain disease
  Alpha heavy chain disease
Plasma cell neoplasms
  Non-IgM MGUS
  Multiple myeloma (plasma cell myeloma)*
    Multiple myeloma, NOS
    Multiple myeloma with recurrent genetic abnormality
    Multiple myeloma with CCND family translocation
    Multiple myeloma with MAF family translocation
    Multiple myeloma with NSD2 translocation
    Multiple myeloma with hyperdiploidy
  Solitary plasmacytoma of bone
  Extraosseous plasmacytoma
Monoclonal Ig deposition diseases
  Ig light chain amyloidosis (AL)*
  Localised AL amyloidosis*
  Light chain and heavy chain deposition disease
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Primary cutaneous marginal zone lymphoproliferative disorder*
Nodal marginal zone lymphoma
  Paediatric nodal marginal zone lymphoma
Follicular lymphoma
In situ follicular neoplasia
Duodenal-type follicular lymphoma
BCL2-R–negative, CD23-positive follicle centre lymphoma
Primary cutaneous follicle centre lymphoma
Paediatric-type follicular lymphoma
Testicular follicular lymphoma*
Large B-cell lymphoma with *IRF4* rearrangement*

Table 20.1a (*Continued*)

Mantle cell lymphoma
  In situ mantle cell neoplasia
  Leukaemic non-nodal mantle cell lymphoma
Diffuse large B-cell lymphoma, NOS
  Germinal centre B-cell subtype
  Activated B-cell subtype
*Large B-cell lymphoma with 11q aberration**
Nodular lymphocyte predominant B-cell lymphoma*
T cell/histiocyte-rich large B-cell lymphoma
Primary diffuse large B-cell lymphoma of the central nervous system
Primary diffuse large B-cell lymphoma of the testis*
Primary cutaneous diffuse large B-cell lymphoma, leg type
Intravascular large B-cell lymphoma
*HHV-8 and Epstein-Barr virus–negative primary effusion-based lymphoma**
Epstein-Barr virus–positive mucocutaneous ulcer*
Epstein-Barr virus–positive diffuse large B-cell lymphoma, NOS
Diffuse large B-cell lymphoma associated with chronic inflammation
  Fibrin-associated diffuse large B-cell lymphoma
Lymphomatoid granulomatosis
Epstein-Barr virus–positive polymorphic B-cell lymphoproliferative disorder, NOS*
ALK-positive large B-cell lymphoma
Plasmablastic lymphoma
HHV-8–associated lymphoproliferative disorders
  Multicentric Castleman disease
  HHV-8–positive germinotropic lymphoproliferative disorder
  HHV-8–positive diffuse large B-cell lymphoma, NOS
  Primary effusion lymphoma
Burkitt lymphoma
High-grade B-cell lymphoma, with *MYC* and *BCL2* rearrangements*
High-grade B-cell lymphoma with MYC *and* BCL6 *rearrangements**
High-grade B-cell lymphoma, NOS
Primary mediastinal large B-cell lymphoma
Mediastinal grey-zone lymphoma*

Classic Hodgkin lymphoma
  Nodular sclerosis classic Hodgkin lymphoma
  Lymphocyte-rich classic Hodgkin lymphoma
  Mixed cellularity classic Hodgkin lymphoma
  Lymphocyte-depleted classic Hodgkin lymphoma

Mature T-cell and NK-cell neoplasms
T-cell prolymphocytic leukaemia
T-cell large granular lymphocytic leukaemia
Chronic lymphoproliferative disorder of NK cells
Adult T-cell leukaemia/lymphoma

Table 20.1a (*Continued*)

Epstein-Barr virus–positive T-cell/NK-cell lymphoproliferative disorders of childhood*
- Hydroa vacciniforme lymphoproliferative disorder
  - Classic
  - Systemic
- Severe mosquito bite allergy
- Chronic active Epstein-Barr virus disease, systemic (T-cell and NK-cell phenotype)
- Systemic Epstein-Barr virus–positive T-cell lymphoma of childhood

Extranodal NK/T-cell lymphoma, nasal type
Aggressive NK-cell leukaemia
Primary nodal Epstein-Barr virus–positive T-cell/NK-cell lymphoma*
Enteropathy-associated T-cell lymphoma
- Type II refractory celiac disease*

Monomorphic epitheliotropic intestinal T-cell lymphoma
Intestinal T-cell lymphoma, NOS
Indolent clonal T-cell lymphoproliferative disorder of the gastrointestinal tract*
Indolent NK-cell lymphoproliferative disorder of the gastrointestinal tract*
Hepatosplenic T-cell lymphoma
Mycosis fungoides
Sezary syndrome
Primary cutaneous $CD30^+$ T-cell lymphoproliferative disorders
Lymphomatoid papulosis
Primary cutaneous anaplastic large cell lymphoma
Primary cutaneous small/medium CD4 T-cell lymphoproliferative disorder
Subcutaneous panniculitis-like T-cell lymphoma
Primary cutaneous gamma-delta T-cell lymphoma
Primary cutaneous acral $CD8^+$ T-cell lymphoproliferative disorder*
Primary cutaneous $CD8^+$ aggressive epidermotropic cytotoxic T-cell lymphoma
Peripheral T-cell lymphoma, NOS
Follicular helper T-cell lymphoma*
- Follicular helper T-cell lymphoma, angioimmunoblastic type (angioimmunoblastic T-cell lymphoma)
- Follicular helper T-cell lymphoma, follicular type
- Follicular helper T-cell lymphoma, NOS

Anaplastic large cell lymphoma, ALK positive
Anaplastic large cell lymphoma, ALK negative
Breast implant–associated anaplastic large cell lymphoma

Immunodeficiency-associated lymphoproliferative disorders
Posttransplant lymphoproliferative disorders
- Nondestructive posttransplant lymphoproliferative disorders
  - Plasmacytic hyperplasia posttransplant lymphoproliferative disorder
  - Infectious mononucleosis posttransplant lymphoproliferative disorder
  - Florid follicular hyperplasia posttransplant lymphoproliferative disorder

Polymorphic posttransplant lymphoproliferative disorder
Monomorphic posttransplant lymphoproliferative disorder (B-cell and T-cell/NK-cell types)†
Classic Hodgkin lymphoma posttransplant lymphoproliferative disorder†
Other iatrogenic immunodeficiency-associated lymphoproliferative disorders

Italic font indicates provisional tumour entities.
* Changes from the 2016 WHO classification.
† These lesions are classified according to the lymphoma to which they correspond.(Source: Campo *et al. Blood*. 2022/with permission of Elsevier)

Table 20.1b WHO-5 edition classification of mature lymphoid neoplasms.

Mature B-cell neoplasms
Pre-neoplastic and neoplastic small lymphocytic proliferations
Monoclonal B-cell lymphocytosis
Chronic lymphocytic leukaemia/small lymphocytic lymphoma

Splenic B-cell lymphomas and leukaemias
Hairy cell leukaemia
Splenic marginal zone lymphoma
Splenic diffuse red pulp small B-cell lymphoma
Splenic B-cell lymphoma/leukaemia with prominent nucleoli (encompassing hairy cell leukaemia variant and some cases of B-cell prolymphocytic leukaemia)

Lymphoplasmacytic lymphoma
Lymphoplasmacytic lymphoma

Marginal zone lymphoma
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue
Primary cutaneous marginal zone lymphoma
Nodal marginal zone lymphoma
Paediatric marginal zone lymphoma

Follicular lymphoma
In situ follicular B-cell neoplasm
Follicular lymphoma
Paediatric-type follicular lymphoma
Duodenal-type follicular lymphoma
Primary cutaneous follicle centre lymphoma

Mantle cell lymphoma
In situ mantle cell neoplasm
Mantle cell lymphoma
Leukaemic non-nodal mantle cell lymphoma

(*Continued*)

Table 20.1b (*Continued*)

Large B-cell lymphomas
Diffuse large B-cell lymphoma, NOS
T-cell/histiocyte-rich large B-cell lymphoma
Diffuse large B-cell lymphoma/ high grade B-cell lymphoma with *MYC* and *BCL2* rearrangements
ALK-positive large B-cell lymphoma
Large B-cell lymphoma with *IRF4* rearrangement
High-grade B-cell lymphoma with 11q aberrations
Lymphomatoid granulomatosis
EBV-positive diffuse large B-cell lymphoma
Diffuse large B-cell lymphoma associated with chronic inflammation
Fibrin-associated large B-cell lymphoma
Fluid overload-associated large B-cell lymphoma
Plasmablastic lymphoma
Primary large B-cell lymphoma of immune-privileged sites, encompassing primary diffuse large B-cell lymphoma of the CNS in revised 4th edition (plus primary large B-cell lymphoma of the vitreoretina and primary large B-cell lymphoma of the testis)
Primary cutaneous diffuse large B-cell lymphoma, leg type
Intravascular large B-cell lymphoma
Primary mediastinal large B-cell lymphoma
Mediastinal grey zone lymphoma
High-grade B-cell lymphoma, NOS

Burkitt lymphoma
KSHV/HHV8-associated B-cell lymphoid proliferations and lymphomas
Primary effusion lymphoma
KSHV/HHV8-positive diffuse large B-cell lymphoma
KSHV/HHV8-positive germinotropic lymphoproliferative disorder

Lymphoid proliferations and lymphomas associated with immune deficiency and dysregulation
Hyperplasias arising in immune deficiency/dysregulation (encompassing non-destructive post-transplant lymphoproliferative disorders, amongst others)
Polymorphic lymphoproliferative disorders arising in immune deficiency/dysregulation (encompassing polymorphic posttransplant lymphoproliferative disorders, other iatrogenic immunodeficiency-associated lymphoproliferative disorders, amongst others
EBV-positive mucocutaneous ulcer
Lymphomas arising in immune deficiency / dysregulation / (encompassing monomorphic posttransplant lymphoproliferative disorders, classic Hodgkin lymphoma posttransplant lymphoproliferative disorders, lymphomas associated with HIV infection, amongst others)

Table 20.1b (*Continued*)

Inborn error of immunity-associated lymphoid proliferations and lymphomas
Lymphoproliferative diseases associated with primary immune disorders

Hodgkin lymphoma
Classic Hodgkin lymphoma
Nodular lymphocyte predominant Hodgkin lymphoma

Plasma cell neoplasms and other diseases with paraproteins
Monoclonal gammopathies
Cold agglutinin disease
IgM monoclonal gammopathy of undetermined significance
Non-IgM monoclonal gammopathy of undetermined significance
Monoclonal gammopathy of renal significance

Diseases with monoclonal immunoglobulin deposition
Immunoglobulin-related (AL) amyloidosis Primary amyloidosis
Monoclonal immunoglobulin deposition disease Light chain and heavy chain deposition disease
Heavy chain diseases
Mu heavy chain disease
Gamma heavy chain disease
Alpha heavy chain disease

Plasma cell neoplasms
Plasmacytoma
Plasma cell myeloma
Plasma cell neoplasms with associated paraneoplastic syndrome

Mature T-cell and NK-cell leukaemias
T-prolymphocytic leukaemia
T-large granular lymphocytic leukaemia
NK-large granular lymphocytic leukaemia
Adult T-cell leukaemia/lymphoma
Sezary syndrome
Aggressive NK-cell leukaemia

Primary cutaneous T-cell lymphomas
Primary cutaneous CD4-positive small or medium T-cell lymphoproliferative disorder
Primary cutaneous acral CD8-positive lymphoproliferative disorder
Mycosis fungoides
Primary cutaneous CD30-positive T-cell lymphoproliferative disorders
Lymphomatoid papulosis
Primary cutaneous anaplastic large cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Primary cutaneous gamma/delta T-cell lymphoma
Primary cutaneous CD8-positive aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous peripheral T-cell lymphoma, NOS

Table 20.1b (*Continued*)

Intestinal T-cell and NK-cell lymphoid proliferations and lymphomas
Indolent T-cell lymphoma of the gastrointestinal tract
Indolent NK-cell lymphoproliferative disorder of the gastrointestinal tract
Enteropathy-associated T-cell lymphoma
Monomorphic epitheliotropic intestinal T-cell lymphoma
Intestinal T-cell lymphoma, NOS
Hepatosplenic T-cell lymphoma
Hepatosplenic T-cell lymphoma
Anaplastic large cell lymphoma
ALK-positive anaplastic large cell lymphoma
ALK-negative anaplastic large cell lymphoma
Breast implant-associated anaplastic large cell lymphoma
Nodal T-follicular helper (TFH) cell lymphoma
Nodal TFH cell lymphoma, angioimmunoblastic-type
Nodal TFH cell lymphoma, follicular-type
Nodal TFH cell lymphoma, NOS
Other peripheral T-cell lymphomas
Peripheral T-cell lymphoma, not otherwise specified
EBV-positive NK/T-cell lymphomas
EBV-positive nodal T- and NK-cell lymphoma
Extranodal NK/T-cell lymphoma
EBV-positive T- and NK-cell lymphoid proliferations and lymphomas of childhood
Severe mosquito bite allergy
Hydroa vacciniforme lymphoproliferative disorder
Systemic chronic active EBV disease
Systemic EBV-positive T-cell lymphoma of childhood

(*Source:* Alaggio *et al*. Leukemia 2022/Springer Nature/CC BY 4.0)

tumour usually involves the peripheral blood and bone marrow, but also lymph nodes, spleen, liver and other extranodal sites. The diagnosis of the disease requires, in the absence of extramedullary tissue involvement, $\geq 5 \times 10^9$/L monoclonal B lymphocytes with a CLL phenotype in the blood, sustained for at least 3 months. The diagnosis may be established with lower cell counts when the patient has disease-related cytopenias or symptoms. The term 'small lymphocytic lymphoma' refers to non-leukaemic neoplasms with the same morphology and phenotype. The presence in blood of a clonal population of B-cells with the CLL phenotype below this number of lymphocytes is called monoclonal B-cell lymphocytosis (MBL). MBL is considered a precursor of CLL, although not all MBL will progress to an overt CLL.

The bone marrow is infiltrated in virtually all cases with an interstitial, nodular or diffuse pattern. The lymph nodes show effacement of the architecture by a diffuse infiltration. Aggregates of prolymphocytes and paraimmunoblasts associated with some follicular dendritic cells and increased numbers of $CD4^+$ T cells, called proliferation centres, are an almost constant feature in the lymph nodes (Figure 20.1). In some cases, these areas are expanded and associated with more aggressive behaviour (accelerated CLL) but should not be diagnosed as transformation to DLBC. (see also Chapter 23).

The mutational status of the immunoglobulin genes (IG) distinguishes two major subtypes of CLL. Thus, 40–50% of cases have unmutated IG (>98% identity with the germline), whereas 50–60% have hypermutated genes. Patients with unmutated-IG CLL have a more aggressive disease. Analysis of immunoglobulin gene sequences has revealed a marked bias in use of the different VH families and the identification of subsets of cases with quasi-identical IGHV sequences called stereotypes, suggesting the influence of certain antigens in the selection of tumour cells. The relevance of the activation of the B-Cell Receptor (BCR) in CLL is highlighted by the identification of the activating mutations R110 in the V3–21 lambda light chain in approximately 7–15% of patients with either mutated or unmutated-IG and conferring an adverse prognosis. The most common chromosomal alterations are deletions of chromosome 13q (50%), trisomy 12 (20%), 11q deletions (15%) and 17p deletions (10%); 13q deletions are more common in mutated CLL, whereas 11q and 17p deletions are more frequent in unmutated CLL and are associated with a worse prognosis. Next-generation sequencing (NGS) studies have identified the complex mutational landscape of the disease that in addition to the frequent *TP53* (3–15%) and *ATM* (10–15%) alterations also include frequent mutations in *NOTCH1* (4–15%), *SF3B1* (5–17%) *BIRC3* (3%), *MYD88* (3%), amongst many others. The distribution of these mutations varies in different subgroups of patients and in the evolution of the disease.

Transformation into a more aggressive tumour occurs in 2–10% of patients (Richter transformation). The two most common forms of transformation are diffuse large B-cell lymphoma (DLBCL) and, less frequently, Hodgkin lymphoma (HL). DLBCL arising in unmutated-IG CLL usually corresponds to the clonal evolution of the preceding CLL, whereas in mutated CLL it frequently corresponds to a different lymphoid neoplasm. DLBCL transformation is frequently associated with *TP53* or *NOTCH1* mutations.

B-cell prolymphocytic leukaemia

B-cell prolymphocytic leukaemia (B-PLL) is a malignancy of B prolymphocytes that affects the blood, bone marrow and spleen and is characterised by more than 55% prolymphocytes

Table 20.2 Immunophenotypic features of common B-cell neoplasms.

Neoplasm	SIG; CIg	CD20	CD5	CD10	BCL6	MUM1/IRF4	CD23	CD43	CD103	Cyclin D1	SOX11	CD38/CD138	CD30	EBV
CLL	+; –/+	+weak	+	–	–	–/+	+	+	–	–	–	–	–	–
Lymphoplasmacytic lymphoma	+; +	+	–	–	–	+	–	+/–	–	–	–	+	–	–
Hairy-cell leukaemia	+; –	+	–	–	–	–	–	+	++	+/–	–/+	–	–	–
Splenic marginal-zone lymphoma	+; –/+	+	–	–	–	–/+	–	–	+	–	–	–	–	–
Follicular lymphoma	+; –	+	–	+/–	+	–/+	–/+	–	–	–	–	–	–	–
Mantle-cell lymphoma	+; –	+	+	–	–	–	–	+	–	+	+	–	–	–
MALT lymphoma	+; +/–	+	–/+	–	–	–/+	–/+	–/+	–	–	–	–	–	–
DLBCL-GCB	+/–; –/+	+	–	+	+	–		–/+		–	–	–	–	–
DLBCL-ABC	+/–; –/+	+	–	–	–/+	+		–	–	–	–	–	–/+	–/+*
PMBL	–	+	–	–	+/–	+/–	+	–	–	–	–	–	+	–
Burkitt lymphoma	+; –	+	–	+	+	–	–	–		–	–/+	–	–	–/+
Plasmablastic lymphoma	+	–	–	–/+	–	+	–	–	–	–	–	+	+/–	+
Multiple myeloma	+	–/+	–	+/–	–	+	–	–/+	–	–/+	–	+	–	–

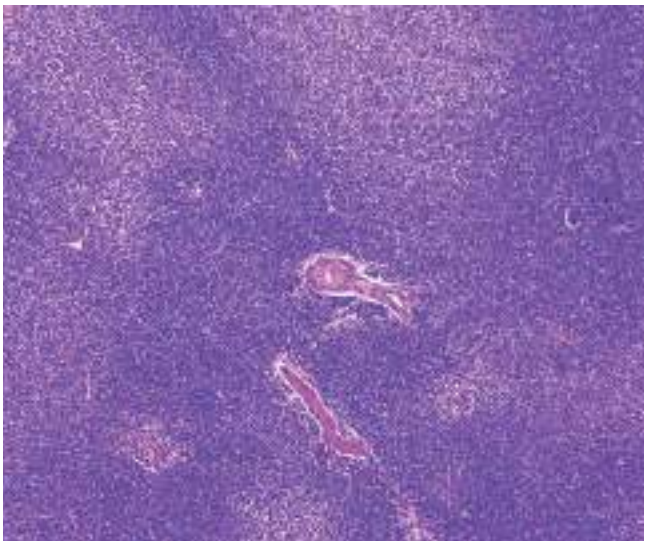
* EBV+ DLBCL
ABC: activated B-cell type; cIG: cytoplasmic immunoglobulin; DLBCL: diffuse large B-Cell lymphoma; EBV: Epstein–Barr virus; GCB: Germinal-centre B-cell type; PMBL: primary mediastinal B-cell lymphoma; sIg: surface immunoglobulin.

in the blood. B-PLL does not include transformed CLL, CLL with increased prolymphocytes, splenic marginal zone lymphomas or blastoid mantle cell lymphoma with t(11;14)(q13;q32), which should be excluded. B-PLL is an uncommon disease of old patients (median age 65–69 years) and similar male/female distribution (see also Chapter 23). Patients have 'B' symptoms, massive splenomegaly, absent or minimal lymphadenopathy and a rapidly rising lymphocyte count, usually over 100×10^9/L. Anaemia and thrombocytopenia are seen in 50%. The cells strongly express surface IgM with or without IgD and mature B-cell antigens. CD5 and CD23 are only positive in 20–30% and 10–20% of cases, respectively. Complex karyotypes are common and *MYC* translocation and 17p deletions associated with *TP53* gene mutations are detected in 40–60% of cases. Median survival is 30–50 months. IGHV somatic hypermutations are present in 50% of the cases but contrary to CLL are not of prognostic value. The ICC maintains the same criteria for this disease but the WHO-HAEM5 does not recognise this disease as a specific entity and assumes that some cases with these characteristics should be included in a new category called 'splenic B-cell lymphoma/leukaemia with prominent nucleoli' together with cases previously diagnosed as 'hairy cell leukaemia variant' – a disease which has also been eliminated in the new WHO-HAEM5 classification.

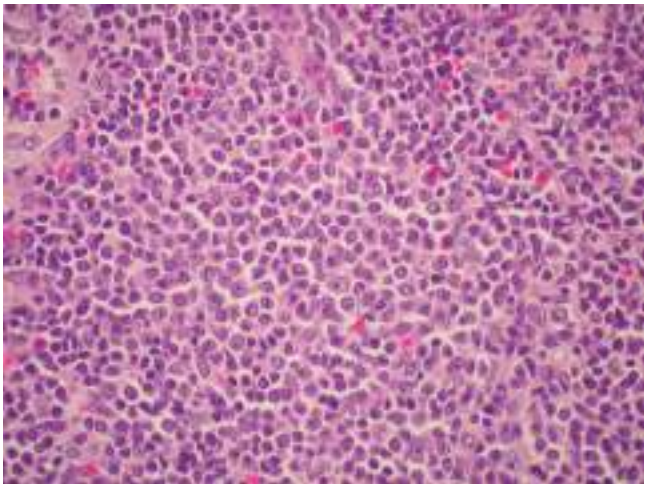
Splenic marginal zone lymphoma

Splenic marginal zone lymphoma (SMZL) is a B-cell neoplasm composed of small lymphocytes that surround and replace the germinal centres and mantle of the reactive follicles in the white pulp, and merge with a peripheral (marginal) zone of larger cells, including scattered transformed blasts. Both small and larger cells infiltrate the red pulp. The patients usually have a leukaemic and splenomegalic presentation with villous lymphocytes in the blood. The disease also involves the splenic hilar lymph nodes and bone marrow, but extension to peripheral lymph nodes is uncommon (Figure 20.2).

There are no distinctive phenotypic markers for SMZL, whose diagnosis requires the exclusion of other lymphoma types (Table 20.2). Most cases exhibit IgM/IgD and a mature B-cell phenotype, but other markers are usually negative (Figure 20.2). The immunoglobulin genes are unmutated in approximately half of the cases, which also tend to have deletions of chromosome 7q31–32 (45%) and a more unfavourable evolution. NGS studies have identified frequent mutations in *KLF2* (10–40%), *NOTCH2*



(a)



(b)

Figure 20.1 Lymph node with chronic lymphocytic leukaemia. (a) At low power the lymph node shows diffuse effacement of the architecture with vaguely nodular pale areas that correspond to the proliferation centres; (b) At higher magnification the proliferation centre contains larger cells with nuclei that have a central nucleolus (prolymphocytes and paraimmunoblasts).

(20–25%) and genes of the NF-κB pathway (*BIRC3*, *TNFAIP3*). A second group of tumours carry mutations in DNA damage, MAP kinase and TLR genes (*TP53*, *ATM, BRAF*). Some cases are positive for hepatitis C virus and may respond to antiviral treatment. The clinical course is usually indolent, but some patients may have progressive disease and transformation to a large-cell lymphoma may occur. In the ICC, the hairy cell leukaemia variant and lymphomas with a diffuse infiltrate of the red pulp are maintained as distinct provisional categories. In the WHO-HAEM5 tumours previously diagnosed as hairy cell leukaemia variant must be included in the new category of 'splenic B-cell lymphoma/leukaemia with prominent nucleoli' that also includes cases that fulfil the criteria of the former B-PLL.

Lymphoplasmacytic lymphoma/Waldenström macroglobulinaemia

Lymphoplasmacytic lymphoma (LPL) is a B-cell neoplasm composed of small lymphocytes, plasmacytoid lymphocytes and plasma cells, usually involving the bone marrow and sometimes lymph nodes and spleen that does not fulfil the criteria for any other B-cell neoplasm which may have plasmacytic differentiation. The ICC has adopted the diagnostic criteria proposed by the International Workshop on Waldenström's macroglobulinaemia. The diagnosis of LPL may be rendered in patients with abnormal lymphoplasmacytic aggregates in the bone marrow and evidence of clonal B cells and plasma cells, even when the aggregates represent <10% of cellularity of the trephine biopsy. Although the detection of a paraprotein is common, it is not required for the diagnosis. Waldenström macroglobulinaemia is an LPL with bone marrow involvement and an IgM monoclonal gammopathy of any concentration. Because these entities do not have specific markers (Table 20.2), it is essential to exclude the presence of any other B-cell neoplasm that may have a plasmacytic differentiation. NGS has identified activating mutations of *MYD88* in 90–100% of the cases and *CXCR4* in 28%. The latter has been associated with development of resistance to ibrutinib therapy. These findings may help in the differential diagnosis of the disease. Primary cold agglutinin disease is now considered a distinct entity from LPL and IgM MGUS in both ICC and WHO-HAEM5 classifications. The disease lacks the $MYD88^{L265P}$ mutation.

Plasma cell neoplasms

Plasma cell neoplasms encompass a spectrum of lesions characterised by the clonal expansion of terminally differentiated B cells that usually secrete a class-switched immunoglobulin (Table 20.3) (see also Chapter 28).

Monoclonal gammopathy of undetermined significance

Monoclonal gammopathy of undetermined significance (MGUS) is defined by less than 10% clonal plasma cells in the bone marrow and less than 30 g/L of an M-protein and absence of end-organ damage. This condition is considered a preneoplastic process since evolution to overt plasma cell malignancy does not always occur. The ICC distinguish two types of IgM MGUS: IgM MGUS of plasma cell type and IgM MGUS, not otherwise specified (NOS). The rare IgM MGUS of plasma cell type is considered a precursor of multiple myeloma (MM) and is defined by clonal plasma cells without a detectable B-cell component and with wild-type *MYD88*. This category also includes patients with t(11;14)(q13;q32) or

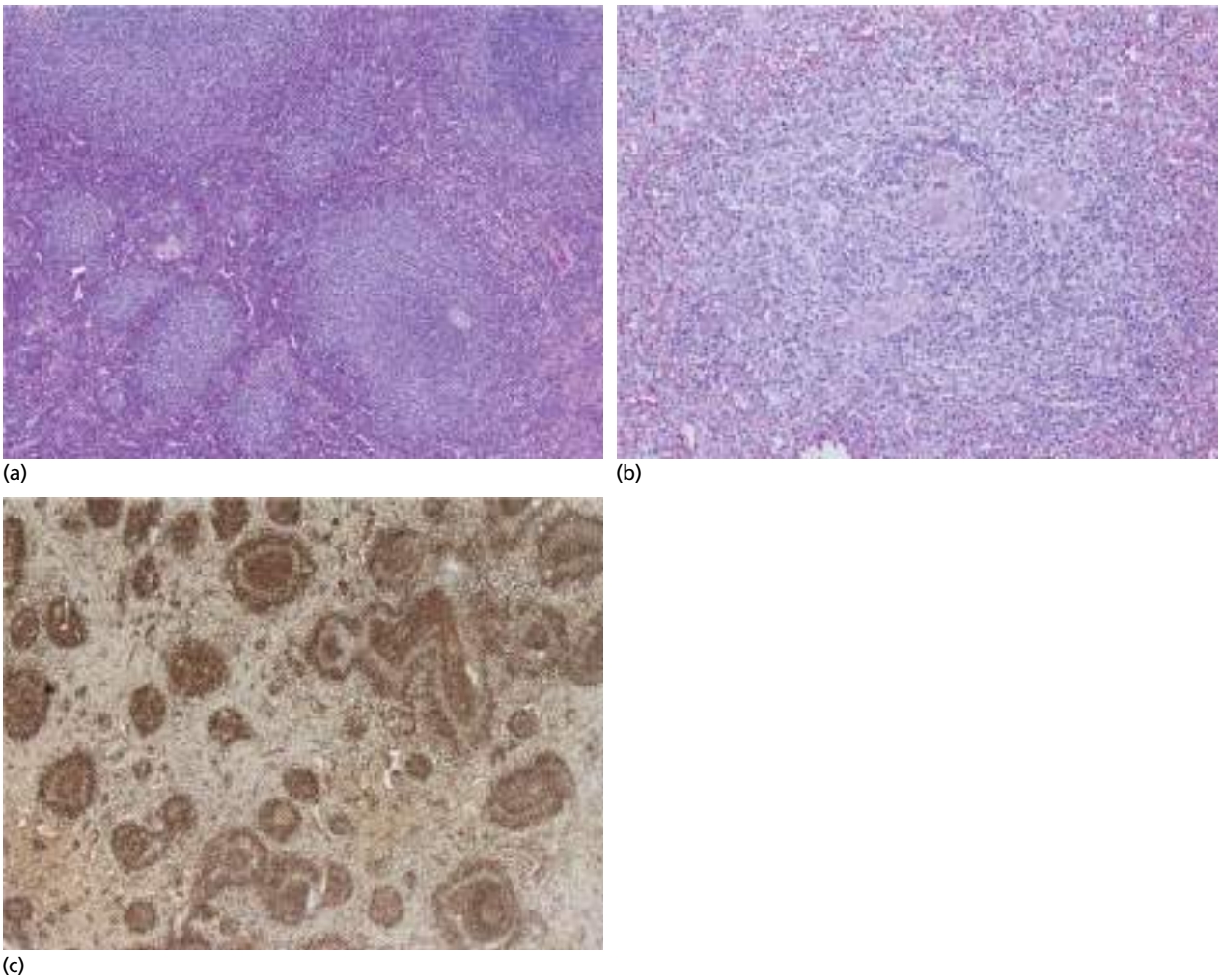


Figure 20.2 Splenic marginal zone lymphoma. (a) The spleen shows multiple nodular proliferations in the red pulp and expanding the white pulp areas; (b) At higher magnification, the nodules are composed of small lymphoid cells in the centre and larger cells at the periphery; (c) The tumour cells are IgD-positive.

Table 20.3 Plasma cell neoplasms.

Non-IgM monoclonal gammopathy of undetermined significance
Plasma cell myeloma
Smouldering (asymptomatic) plasma cell myeloma
Non-secretory myeloma
Plasma-cell leukaemia
Plasmacytoma
Solitary plasmacytoma of bone
Extraosseous (extramedullary) plasmacytoma
Monoclonal immunoglobulin deposition diseases
Plasma cell neoplasms with associated paraneoplastic syndrome
Osteosclerotic myeloma (POEMS syndrome)
TEMPI syndrome

other cytogenetic abnormalities typical of MM. The IgM MGUS, NOS includes all those with a *MYD88* mutation, those with detectable monotypic or monoclonal B cells by flow cytometry but without abnormal lymphoplasmacytic aggregates diagnostic of LPL, and those who lack evidence of other small B-cell neoplasms.

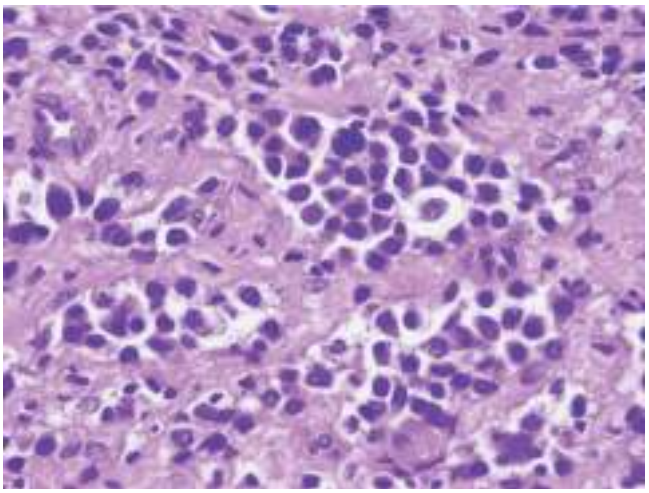
Multiple myeloma (Plasma cell myeloma)

The ICC preferred the term multiple myeloma over the previous WHO of plasma cell myeloma. It is a bone marrow neoplasm characterised by multifocal proliferation of plasma cells associated with a serum M-protein and symptomatic disease related to different organ dysfunction or lytic bone lesions. The plasma cells in the bone marrow expand in small or large clusters. Some cases may present asymptomatically, whereas in others, the

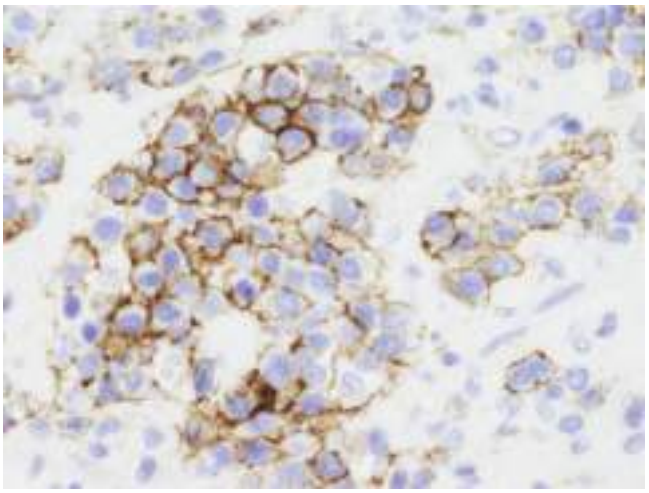
tumour cells spread to the peripheral blood in the form of a plasma-cell leukaemia. The phenotype of these cells is aberrant, with expression of CD19 and CD56 in 75% of cases and other markers such as CD117, CD20, CD52 and CD10. The ICC recommended the subclassification of this disease based on the presence of cytogenetic alterations including MM, NOS when no specific alterations are found and MM with recurrent genetic abnormalities including MM with CCND family translocations, MM with MAF family translocation, MM with NSD2 translocation and MM with hyperdiploidy. Detection of t(4;14), t(14;16) and secondary changes, including del(17p), amp1q and del(1p) identifies patients with high-risk disease. *MYC* translocations have been identified in around 20% of cases, also associated with plasmablastic features.

Plasmacytoma

Plasmacytoma is a solitary tumour lesion composed of clonal plasma cells that may occur in bones or extraosseous tissues (Figure 20.3). Bone plasmacytomas evolve to plasma cell myeloma in two-thirds of patients. In contrast, extraosseous localised lesions have a relatively indolent behaviour without bone marrow involvement, suggesting a closer relationship to MALT lymphomas than bone marrow plasma-cell neoplasms. Progression of solitary plasmacytomas to multiple myeloma is frequently associated with presence of clonal plasma cells in the bone marrow. The ICC recommends the study of bone marrow in all cases because minimal marrow involvement detected by flow cytometry (i.e. clonal plasma cells present but <10%) is associated with higher risk of progression, particularly with solitary plasmacytomas of bone.



(a)



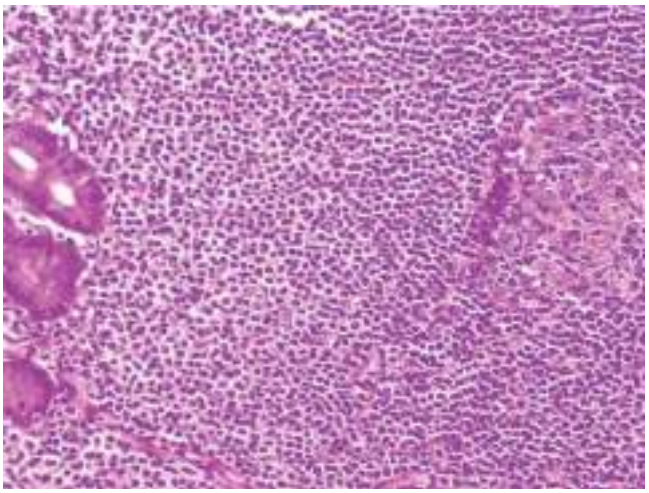
(b)

Figure 20.3 Plasmacytoma of the nasal cavity. (a) Atypical plasma cells embedded in fibrous stroma; (b) Plasma cells are CD138 positive.

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)

MALT lymphomas are extranodal B-cell neoplasms composed of a heterogeneous population of small lymphocytes, marginal zone lymphocytes with cleaved nuclei (centrocyte-like), cells with clear cytoplasm resembling monocytoid B cells, cells with plasmacytic differentiation and occasional large, transformed cells. All these cells expand the marginal zone of reactive follicles and in some cases may colonise the germinal centres but preserve the mantle zone area. In epithelial tissues, the neoplastic cells infiltrate the epithelium forming lymphoepithelial lesions (Figure 20.4). These tumours occur most commonly in the gastrointestinal tract, salivary gland, lung, head and neck, ocular adnexa and, less frequently, thyroid and breast. Both ICC and WHO-HAEM5 consider that the primary cutaneous MZL is a different entity than MZL in other locations and the ICC proposed to name these cases as primary cutaneous lymphoproliferative disorder, instead of lymphoma, to highlight the very indolent behaviour of these cases. It recommends also to distinguish cases with IG class-switched from those expressing IgM, with the later requiring more caution for its possible associated with systemic disease.

The lymphoma cells express mature B-cell markers, IgM and less often IgG or IgA, but IgD is usually negative. CD5, CD10 and CD23 are negative, although some CD5-positive cases have been described (Table 20.2). Four major translocations have been associated with these lymphomas, t(11;18), t(1;14), t(14;18) and t(3;14), that generate the chimeric gene *BIRC3–MALT1* or activate *BCL10*, *MALT1* and *FOXP1*, respectively. The t(11;18) translocation tends to occur more frequently in gastric and lung lymphomas, whereas the t(14;18) translocation is more often seen in salivary gland and ocular adnexa tumours. Interestingly, *MALT1* and *BCL10* participate in activation of the same NF-κB pathway.



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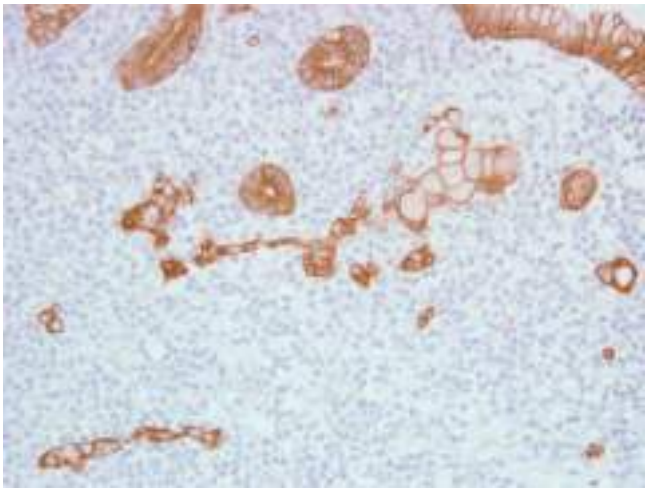


(b)

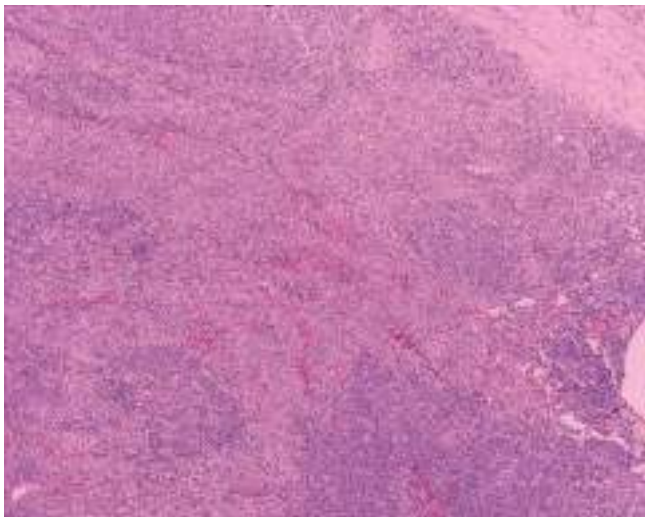
Figure 20.4 Gastric marginal zone lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma). The gastric mucosa is infiltrated by a monotonous population of lymphoid cells that surrounds a residual germinal centre (a) and destroys the glands, creating lymphoepithelial lesions clearly observed with cytokeratin staining (Cam 5.2 immunoperoxidase staining) (b).

These lymphomas arise in topographic sites with a pre-existing chronic inflammatory lesion induced by infectious, immunological or unknown stimuli. Thus gastric lesions are associated with *Helicobacter pylori*, *Campylobacter jejuni* is detected in the immunoproliferative small intestinal disease, *Chlamydia psittaci* in ocular adnexa tumours of certain geographic regions and *Borrelia burgdoferi* in some cutaneous MALT lymphomas. Autoimmune disorders of the salivary gland (Sjögren syndrome) and thyroid (Hashimoto disease) are the preceding lesions of MALT lymphomas in these locations.

The lymphoma responds to control of the underlying infectious disease and local treatments but may relapse in extranodal territories after many years. Nodal dissemination may precede clinically the detection of extranodal involvement. Tumours with the translocations described appear to be resistant to antibiotic therapy. Transformation to large-cell lymphomas may occur.

Nodal marginal zone lymphoma

Nodal marginal zone lymphoma (NMZL) is a primary lymphoma of the lymph nodes that resembles extranodal MALT lymphomas (Figure 20.5). An extranodal or splenic lymphoma should be ruled out before this diagnosis is established. Although the morphology and phenotype are similar to MALT



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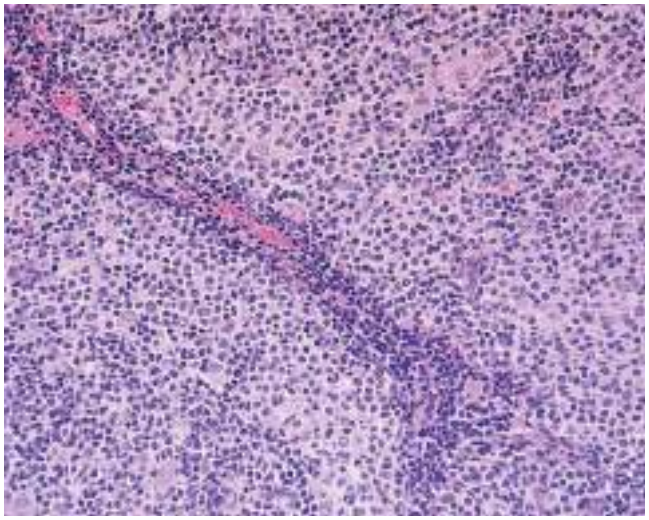


(b)

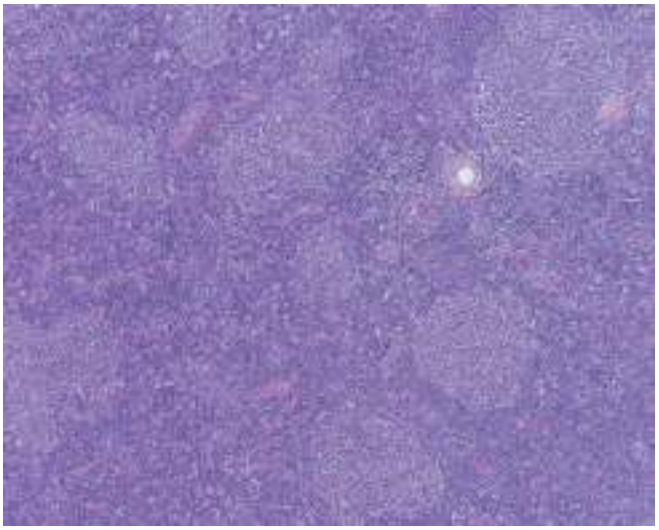
Figure 20.5 Nodal marginal zone lymphoma. (a) Tumour cells with a clear appearance infiltrate the node, with partial preservation of the architecture; (b) Higher magnification reveals the clear cytoplasm of the tumour cells.

lymphoma, primary NMZL does not exhibit the typical translocations of these tumours but have trisomy 3 and 18. The mutational profile is similar to that of SMZL, with frequent mutations in *KLF2, NOTCH2, TNFAIP3, KMT2D* and *TP53*. Disseminated disease may present in some patients and 60–80% survive more than 5 years. Some cases may evolve to large B-cell lymphomas.

NMZLs in the paediatric age group seem to have distinctive clinical and morphological features. Thus, they present predominantly in males as asymptomatic localised tumours and morphologically the residual follicles may have features of progressive transformed germinal centres with eroded mantle zones by the tumour cell infiltration. These tumours may be a morphological variant of paediatric type follicular lymphoma. Molecular and genetic studies detect clonal rearrangements of the IG or chromosomal alterations in virtually all cases and mutations similar to paediatric type follicular lymphoma. These findings are useful in the differential diagnosis with reactive hyperplasias. The prognosis of these patients seems excellent with conservative therapy.

Follicular lymphoma

Follicular lymphoma (FL) is a neoplasm composed of cells of the germinal centres, with different proportions of small centrocytes and large centroblasts, that usually has a follicular growth pattern (Figure 20.6). These tumours are common in Western countries, accounting for around 20–30% of all lymphomas. These tumours have been graded histologically according to the



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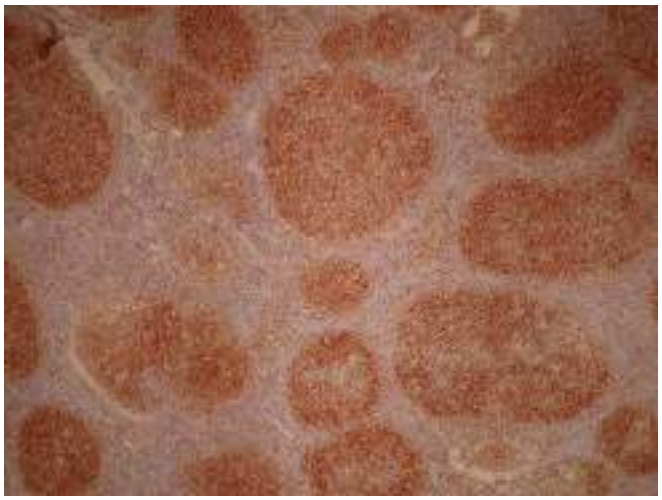

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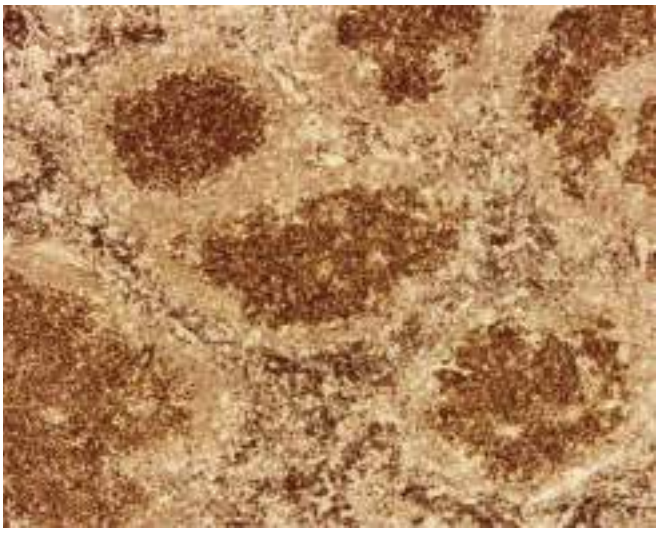


(c)

Figure 20.6 Follicular lymphoma. (a) The tumour grows in a follicular pattern with expanded germinal centres without macrophages. The tumour cells are positive for CD10 (b) and BCL2 (c).

number of large cells, as follows: grade 1 (0–5 large cells per high-power field), grade 2 (6–15 large cells) and grade 3 (>15 large cells). Grades 1 and 2 represent a morphological continuum that is not associated with relevant clinical or biological differences and therefore these cases are grouped together. However, grade 3 tumours are further distinguished into grades 3A and 3B according to the presence of intermingled small centrocytes in grade 3A, whereas grade 3B is composed entirely of large cells. FL grade 3A is genetically and phenotypically more related to FL grade 1–2, whereas FL grade 3B is closer to DLBCL. Any diffuse area composed of more than 15 large cells should be reported as DLBCL, and the percentage of the respective DLBCL and FL components indicated. The ICC maintains these criteria but the WHO-HAEM5 considers grading of FL as optional and denominates FL grade 3B as follicular large B-cell lymphoma. The WHO-HAEM5 also recognises a FL with unusual features characterised by blastoid cells or large centrocyte. The ICC recognises that some high-grade FL may be difficult to classify but recommends that cases with *BCL2* rearrangements and or CD10 expression be included in the group of grade 3A whereas the cases without these features may be closer to FL grade 3B.

FL has a mature B-cell phenotype with coexpression of the germinal-centre markers CD10, BCL6 and LMO2 (Figure 20.6) (Table 20.2). CD5, CD43 and CD23 are negative. BCL2 is positive in 85–90% of FL grade 1–2, but only in 50% of grade 3. BCL2 staining is very useful because reactive germinal centres are negative (Figure 20.6). This expression reflects the presence of the t(14;18) translocation that is the genetic hallmark of this lymphoma and which targets the *BCL2* gene. FL grade 3B is less frequently positive for CD10 and BCL2 protein and the t(14;18) translocation is only detected in 5–20% of cases. In contrast, these lymphomas express IRF4/MUM1 in 40% of the cases and carry 3q27 and *BCL6* rearrangements in 30–50% of cases, whereas these aberrations are rare in FL grades 1–3A. The strong expression of IRF4/MUM1 in grade 3 FL, particularly in young patients, should rise the possibility of large B-cell lymphoma with *IRF4* rearrangement. Some cases may progress to a DLBCL and this transformation may be associated with *TP53* mutations, *CDKN2A/B* homozygous deletions or t(8;14) translocations involving *MYC*.

The tumour cells in FL are associated with a rich microenvironment of different types of T cells and histiocytes that seem to play a major role in determining the biological behaviour of the lymphoma. NGS studies in FL have identified frequent mutations in genes involved in chromatin remodelling, particularly in *KMT2D* (90%), *CREBB* (40%) and *EZH2* (20%).

In situ follicular neoplasia

In situ follicular neoplasia is a variant in which the tumour cells are limited to the germinal centres of the follicles. Usually, only a number of the lymph node follicles are involved, whereas others are reactive. The tumour cells are recognised by their strong BCL2 and CD10 expression inside the germinal centres. These cells carry the t(14;18) translocation. Some of these cases may correspond to early involvement of a lymph node by a disseminated tumour. A second group of patients may develop overt FL during follow-up, but most patients (94%) remain free of lymphoma elsewhere after many years of follow-up.

BCL2-R negative, CD23-positive follicle centre lymphoma

The ICC recognises this category as a provisional entity as a lymphoma derived of follicle centre cells that may grow in a predominant diffuse pattern and lacks *BCL2* rearrangement. These cases usually present with large masses in inguinal or pelvic areas. The tumour expresses CD10, CD23 and carries del(1p36). The mutational profile is different from conventional FL since they have frequent mutations in *STAT6* and lack mutations of the conventional FL such as *EZH2*. These cases partially overlap with the diffuse variant of FL in the WHO-HAEM5. However, 30% of cases *BCL2*-R negative and CD23-positive with the same mutational profile are purely nodular.

Primary intestinal follicular lymphoma

Primary intestinal FL occurs frequently in the duodenum as an incidental finding (Figure 20.7). These cases have a conventional morphology, phenotype and genetic features, including the t(14;18). However, the tumours express IgA and remain localised, with an excellent prognosis, even without treatment.

Primary cutaneous follicle centre lymphoma

Primary cutaneous follicle centre lymphoma generally presents in the skin of the head and trunk. The tumour cells may grow with a follicular or more diffuse pattern. The cells express B-cell

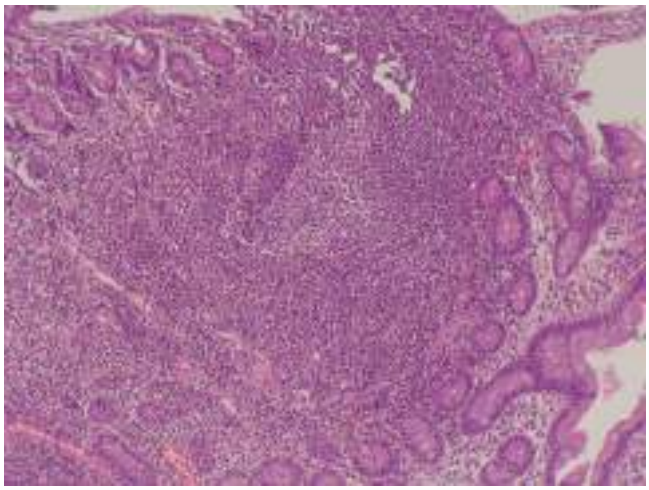


Figure 20.7 Follicular lymphoma of the duodenum.

markers and BCL6, whereas CD10 is positive in cases with a follicular pattern but tends to be lost in the diffuse component. BCL2 expression and the t(14;18) translocation are usually negative. The tumours may relapse, but have a good outcome without extracutaneous dissemination, even with only localised therapy.

Paediatric type follicular lymphoma

Paediatric type FL occurs in children and young adults, usually involving the head and neck region. The ICC recognises Testicular FL as a distinct category, usually in children with localised disease. The lymphomas are BCL2 negative, do not carry the t(14;18) translocation and are composed of large cells with blastoid nuclei. These cases have a good evolution with local therapy. These tumours have mutations in *MAP2K1*, *IRF8*, deletions and mutations of *TNFRS14* and lack mutations typical of adult FL.

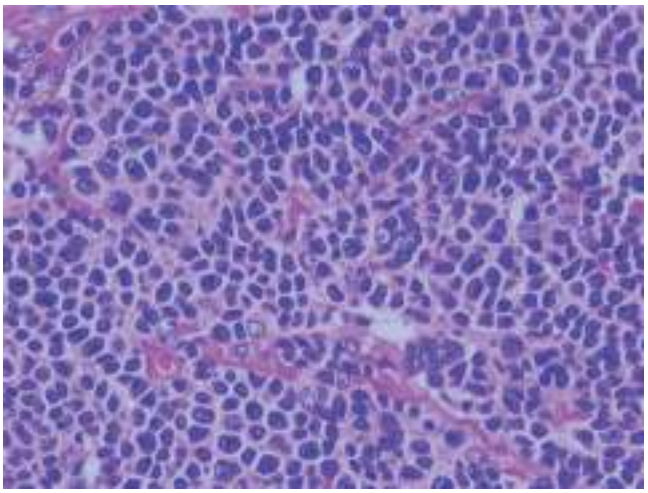
Large cell lymphoma with *IRF4* translocation

These tumours are derived from germinal centre cell and may have a follicular, follicular and diffuse or diffuse pattern. The tumour cells express germinal centre markers with strong IRF4/MUM1 due to and *IRF4* translocation with *IG* genes. The tumours present in the Waldeyer's ring, head and neck and abdominal nodes.

Mantle-cell lymphoma

Mantle-cell lymphoma (MCL) is a B-cell neoplasm composed of monomorphous small- to medium-sized lymphoid cells with irregular nuclear contours and cyclin D1 over-expression secondary to *CCND1* translocation (Figures 20.8 and 20.9). This tumour comprises 3–10% of non-Hodgkin lymphomas and occurs mainly in males, with a median age around 60 years. Most MCLs are diagnosed as disseminated nodal disease, but a number of patients are initially seen with leukaemic disease, bone marrow involvement and frequently splenomegaly, but without lymphadenopathy (leukemic non-nodal MCL). Extranodal involvement is very common. Some tumours may have a blastoid or pleomorphic cytology resembling lymphoblasts or DLBCL, respectively. These cytological variants are associated with higher proliferative activity, more complex karyotypes and worse prognosis. The tumour cells grow with a vaguely nodular, diffuse or mantle-zone pattern. Some cases may show restricted infiltration to the inner mantle zones of otherwise reactive follicles, a situation that has been named *in situ* mantle cell neoplasia. The tumour cells express mature B-cell markers, with intense CD20 and surface immunoglobulin, and coexpress CD5 and CD43. CD23 and germinal-centre markers are negative (Table 20.2).

MCL exhibits the t(11;14) translocation that targets *CCND1*. Some MCL cyclin D1 negative cases carry translocations and overexpression of CCND2 and CCND3. SOX11 is highly



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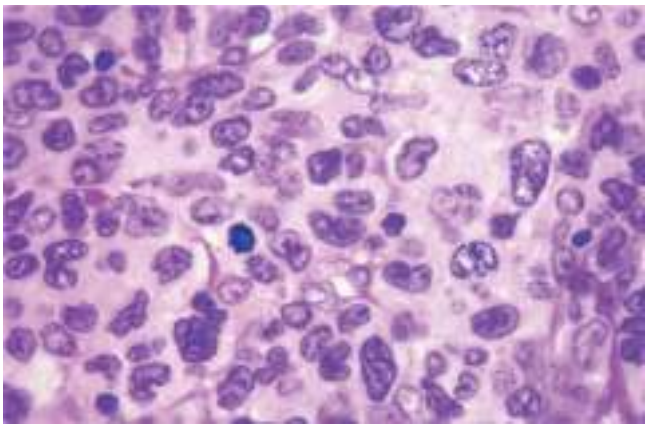


(b)

Figure 20.8 Mantle-cell lymphoma with classical (a) and pleomorphic (b) morphology.

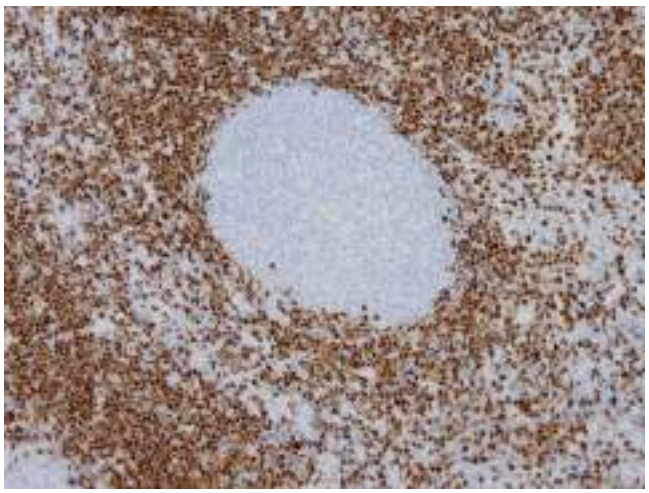


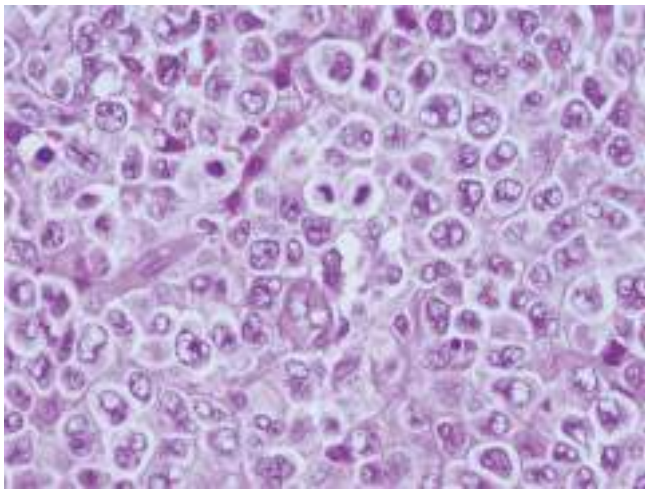
Figure 20.9 Mantle-cell lymphoma is positive for cyclin D1.

expressed in MCL but not in other mature B-cell lymphomas, with the exception of Burkitt lymphoma (30%). SOX11-positive tumours usually have unmutated IGHV and accumulate complex karyotypes. Leukaemic non-nodal MCL is a distinct molecular variant that does not express SOX11, has hypermutated IGHV and simple karyotypes. NGS has identified frequent mutated genes in conventional MCL including *ATM* (41%), *TP53* (28%), *NOTCH1/2* (10%), *KMT2D* (15%) *NSD2* (10%), *MEF2B* (3%) and activation of the NF-κB pathway (*BIRC3*, *TRAF2/3*) (6%). SOX11-negative tumours have *TP53* mutations associated with worse prognosis but usually do not have mutations in the other genes.

In general, MCL has an aggressive clinical course, with frequent relapses after responding to chemotherapy and a median survival of 3–5 years but it is improving with the new therapies. Proliferation is considered the best biological prognostic parameter. Leukaemic non-nodal MCL have an initial indolent disease and better prognosis although may progress to more aggressive behaviour usually associated with *TP53* mutations.

Diffuse large B-cell lymphoma, not otherwise specified

DLBCL is a neoplasm of large B lymphocytes with a diffuse growth pattern. These lymphomas are heterogeneous and several morphological variants, phenotypical and molecular subtypes and different entities have been recognised (Table 20.1b). DLBCL accounts for 25–30% of adult non-Hodgkin lymphomas. Usually, these cases present as primary tumours, but they can also represent the transformation of a less aggressive B-cell lymphoma. Morphologically, DLBCL not otherwise specified (NOS) may have centroblastic, immunoblastic or anaplastic cytology (Figures 20.10 and 20.11). Phenotypically, DLBCL, NOS expresses mature B-cell markers. CD5 is detected in a subset of these tumours. The expression of the germinal-centre markers CD10, BCL6 and LMO2 define a germinal-centre origin, whereas the expression of IRF4/MUM1 and FOXP1 has been associated with an origin in non-germinal-centre activated B cells (Figures 20.10 and 20.11). Although with some limitations, these two immunophenotypic subgroups of DLBCL correlate with the two molecular subtypes of germinal-centre (GCB) and activated B-cell (ABC) DLBCL, recognised by gene expression profiling. These two major molecular subtypes differ in genetic, molecular and clinical aspects, indicating that they correspond to different biological entities. ABC, but not GCB, DLBCL exhibits constitutive activation of the NF-κB pathway. The clinical outcome is also different, with 5-year survival rates of approximately 60 and 30% in GCB and ABC DLBCL, respectively, with current therapies. NGS has identified up to 7 genetic subtypes of DLBCL (Table 20.4) that are characterised by different mutated genes and/or chromosomal alterations. These subgroups are preferentially included in the GCB, ABC or unclassified gene expression-based molecular subtypes, but there are some overlaps. The prevalence of the different subgroups varies from 2 to 18% of the cases and the 5-year overall survival is also very variable from 27 to 84% of the patients. In addition to the prognostic interest of these subgroups, they may be of interest for future targeted therapies. A paradigmatic group is the so-called MCD/C5 group that carries frequent mutations in *MYD88*/*CD79B*. Cases in this group are mainly extranodal in location such as primary DLBCL of CNS and testis, primary cutaneous DLBCL, leg type and intravascular large B-cell lymphoma amongst others. Another interesting group is the EZB MYC$^+$ that has a germinal centre phenotype with *BCL2* and *MYC* rearrangement, that currently is pathologically classified as high-grade B-cell lymphoma with *BCL2* and *MYC* rearrangement. These cases have very aggressive behaviour.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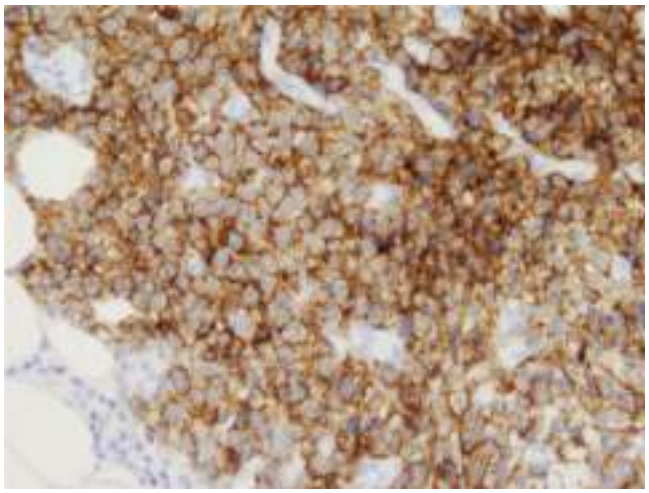
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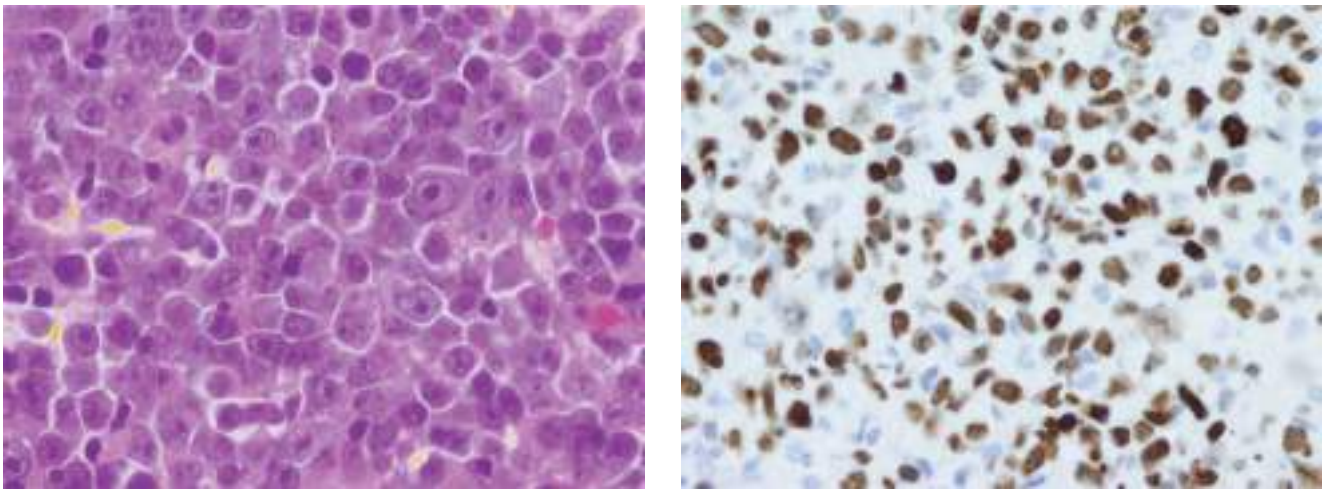
Figure 20.10 Diffuse large B-cell lymphoma (a) positive for CD10 (b) suggests a germinal-centre cell origin.



(a) (b)

Figure 20.11 Diffuse large B-cell lymphoma with immunoblastic morphology (a) and positivity for MUM1/IRF4 (b) suggesting a non-germinal-centre cell origin.

Table 20.4 Genetic subgroups of DLBCL.

Genetic subgroup	Consensus clustering	Prevalence	Cell of origin	Mutations and genetic alterations	Overall survival (5yr)
MCD	C5	9–14%	ABC 99%	*MYD88/CD79B PIM1*	40–42%
N1		2%	ABC94%	*NOTCH1*	27%
A53	C2	7–21%	ABC76%	*TP53 Aneuploidy*	63%
BN2	C1	16–22%	ABC40% UC 42%	*BCL6-R* *NOTCH2*	48–67%
ST2	C4	5–17%	GCB 56% UC 22% ABC22%	*SOCS1, JAK/STAT*	65–84%
EZB MYC+	C3	6%	GCB 95%	*EZB BCL2-R/ MYC-R, DDX3X*	48%
EZB MYC–	C3	13–18%	GCB 95%	*EZB BCL2-R TNFAIP3, CARD11*	82%

(*Source:* Adapted from Wright GW, Huang DW, Phelan JD, *et al.* (2020) and Chapuy B, Stewart C, Dunford AJ, *et al.* (2018).

T-cell/histiocyte-rich large B-cell lymphoma

T-cell/histiocyte-rich large B-cell lymphoma is characterised by a limited number of scattered large B cells immersed in a rich background of T cells and frequently histiocytes. The tumour cells express mature B-cell markers and frequently BCL2 and EMA, although CD30, CD15 and CD138 are negative. The background is composed of CD3 and CD4 T cells and CD68-positive histiocytes. T-cell rosettes surrounding tumour B cells are not seen. Cases positive for Epstein–Barr virus (EBV) and a similar morphology must be classified as EBV-positive DLBCL, NOS. Recent studies suggest that this lymphoma may be closely related to nodular lymphocyte-predominant Hodgkin lymphoma. Patients present with disseminated disease involving lymph nodes but also spleen, liver and bone marrow. Failure of therapy and International Prognostic Index (IPI) score are predictors of survival.

Primary mediastinal large B-cell lymphoma

Primary mediastinal (thymic) large B-cell lymphoma (PMBL) originates in a thymic B cell and predominates in young women presenting with a large mediastinal mass that frequently invades adjacent structures. Progression outside the mediastinum frequently involves extranodal sites such as kidney, liver, adrenal or central nervous system (CNS). The tumour is mainly composed of large cells with abundant pale cytoplasm, which express mature

B-cell markers, but usually lack surface immunoglobulin. CD30 is positive in 80% of cases, although not as uniformly as in HL. CD23 is also frequently positive. CD15 is negative. (Table 20.2) Genetically, PMBL has frequent gains and amplifications of 9p24 including *PDL1*, *PDL2* and *JAK2*. Frequent mutations involving genes and pathways similar to those in Hodgkin lymphoma and mediastinal grey zone lymphomas including JAK-STAT, NF-κB and immune escape pathways (e.g. *SOCS1*, *STAT6*, *NFKBIE* and *CIITA*) are observed. These tumours also carry translocations of the MHC class II transactivator *CIITA* and the immunoresponse modulator *PDL1* and activating mutations of the phosphatase *PTPN1*. Intriguingly, all these alterations may also be seen in a proportion of Hodgkin lymphomas highlighting the genetic relationship between these entities that is also seen in the gene expression profile. Some patients may present with combined HL and PMBL at diagnosis or relapse or have tumours with intermediate features of both (see below). The outcome of these tumours is more favourable than other DLBCL, with a 5-year survival of 65%.

DLBCL with a predominant extranodal location

Several DLBCL subtypes are characterised by extranodal presentation. Intriguingly, although the particular topographic site of these entities configure a unique clinical presentation all of them share a similar *ABC* gene expression profile and landscape of somatic mutations defined in the MCD/C5 genetic subgroups with mutations in *MYD88* and *CD79B*, that suggest they may correspond to the same entity with particular differences related to the site of origin.

Primary DLBCL of the central nervous system (CNS)

This lymphoma originates in the brain, spinal cord, leptomeninges or eye and may appear in patients with a broad range of age. The tumour cells are large with a non-GCB phenotype (*IRF4*/*MUM1* and *BCL6*) and frequently have molecular alterations that favour immune escape (e.g. deletion of HLA). EBV is negative. Sequencing studies have shown frequent mutations in *MYD88, CD79B* and *PIM*.

Primary diffuse large B-cell lymphoma of the testis

Both the ICC and WHO-HAEM5 recognise these tumours as a distinct entity biologically related to primary DLBCL of the CNS. These tumours are mainly of the ABC subtype with frequent mutations in *MYD88/ CD79b* similar to the MCD/C5 genomic subtypes.

Intravascular large B-cell lymphoma

This lymphoma is a distinctive entity characterised by the growth of large B cells within the lumina of small- to medium-sized vessels and capillaries (Figure 20.12). It is relatively rare in Western countries, but more common in Eastern populations.

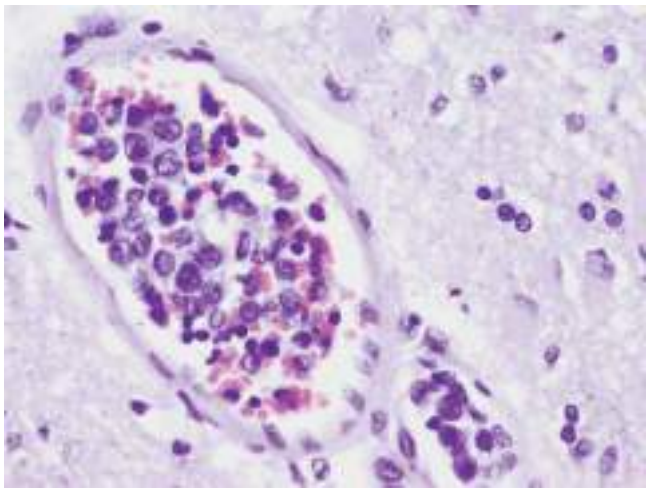


Figure 20.12 Intravascular lymphoma in the brain.

This lymphoma is very aggressive and frequently diagnosed only at postmortem. An isolated cutaneous variant with better prognosis has been identified, mainly in women.

Primary cutaneous DLBCL, leg type

Primary cutaneous DLBCL, leg type, is composed almost exclusively of atypical large B cells that commonly, but not exclusively, present in the lower extremities. These tumours express an activated B-cell phenotype with positivity for IRF4/MUM1 and CD10 negativity. BCL6 is frequently positive, and BCL2 is strongly expressed. The prognosis is poor and the tumour tends to disseminate to extracutaneous sites.

Large-cell lymphomas of terminally differentiated B cells

A group of large B-cell lymphomas that have in common the proliferation of large lymphoid cells with a terminally differentiated B-cell phenotype characterised by variable or total lack of CD20 expression, and less frequently CD79a, but which express plasma-cell-associated antigens such as CD38 and CD138 (Table 20.2). Most of these tumours are infected with EBV or human herpesvirus (HHV)-8 and occur in patients with a certain immunodeficiency status.

ALK-positive large B-cell lymphoma

ALK-positive large B-cell lymphoma is characterised by proliferation of large B cells with plasmablastic differentiation, CD30 negative, that express ALK and are EBV negative. These tumours present in young immunocompetent patients, usually with nodal involvement and aggressive behaviour. ALK expression is due to *ALK* rearrangements, particularly with clathrin [t(2;17)] or less frequently nucleophosmin [t(2;5)]. Contrary to other lymphomas in this group, viral infection and immunodeficiency are absent.

Plasmablastic lymphoma

Plasmablastic lymphoma is a large B-cell lymphoma with frequent immunoblastic morphology and plasma-cell immunophenotype (Table 20.2) that presents mainly in extranodal sites such as oral mucosa and gastrointestinal tract of immunosuppressed patients. HIV infection is the main cause, but post-transplant or immunosuppressive treatments are also common causes. Most of the tumours are EBV positive and latent membrane protein (LMP)-1 negative. The clinical behaviour is aggressive, with poor response to therapy. *MYC* is rearranged in around 50% of the cases. NGS studies have demonstrated a specific profile with frequent mutations in *TP53*, JAK/STAT3 and MAPK (*NRAS*, *KRAS*) pathway genes.

Primary effusion lymphoma

Primary effusion lymphoma occurs mainly in immunosuppressed patients associated with HHV-8 and, frequently, EBV infection. It is characterised by proliferation of large pleomorphic B cells that lack expression of B-cell markers and immunoglobulin, but express plasma-cell-associated antigens and CD30 (Figure 20.13). These tumours usually present as effusion lymphomas, with no involvement of tissues. However, some patients may present with, or later develop, a solid tumour in extranodal sites.

Burkitt lymphoma

Burkitt lymphoma (BL) is mainly a tumour of children and young adults characterised by monotonous proliferation of medium-sized B cells with a mature B and germinal-centre phenotype, negative or very weak BCL2 expression, high proliferation (Ki-67 >95%) and the t(8;14) translocation with *MYC* rearrangement. These tumours occur endemically in equatorial Africa and other geographic areas and sporadically throughout the world. BL is also seen associated with immunodeficiency, particularly HIV infection. The tumour mainly involves extranodal sites, particularly the abdominal cavity, and, in endemic areas, jaws and facial bones. The tumour may infiltrate the CNS. A leukaemic phase may be seen in patients with bulky disease. Occasional patients may present with a pure leukaemic disease (Burkitt leukaemia variant) but in these cases the *MYC* rearrangement seems to be acquired in precursor cells and should be considered a form of acute lymphoblastic leukaemia. The tumour is clinically aggressive but potentially curable with current protocols.

Morphologically, the tumour cells are very monomorphic, highly proliferative, associated with a 'starry sky' pattern of histiocyte phagocytosing apoptotic bodies (Figure 20.14). Genetically, the t(8;14) translocation is found in most tumours with very few if any additional genetic alterations. NGS studies

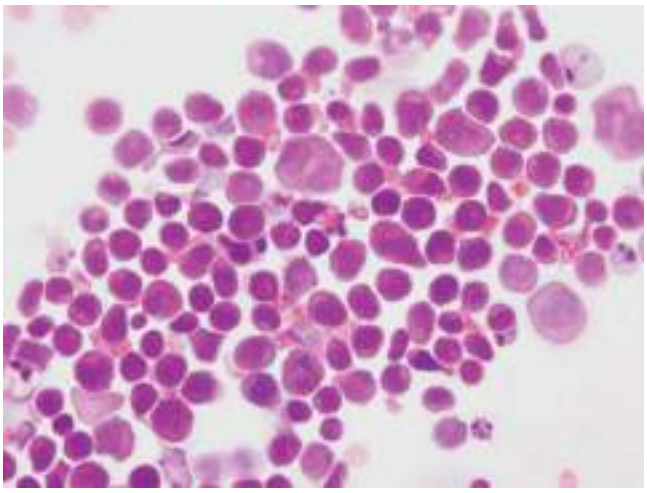
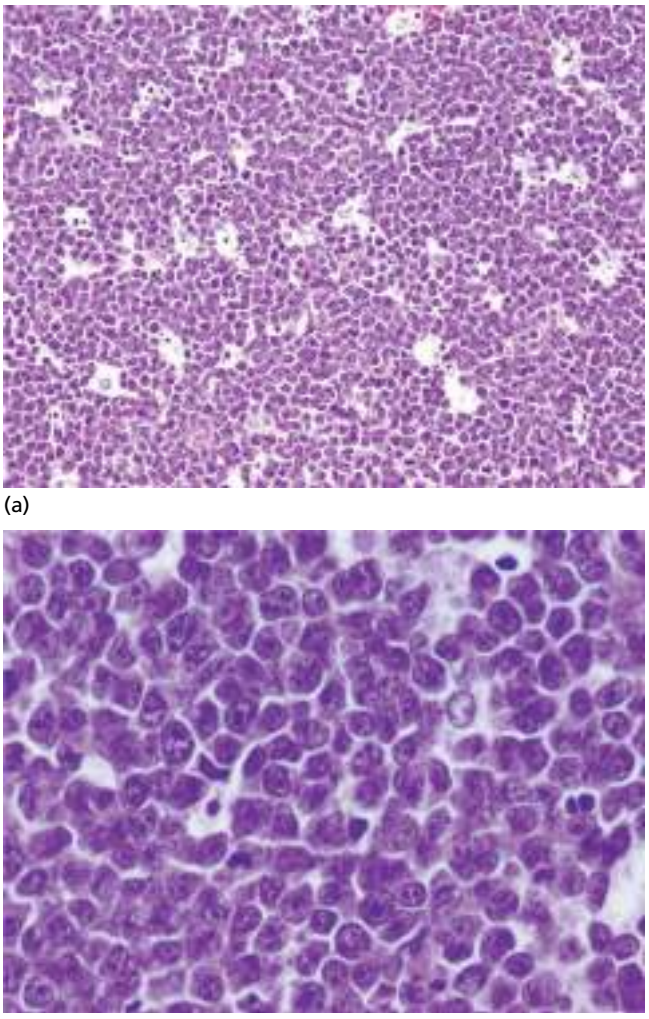


Figure 20.13 Primary effusion lymphoma. The tumour cells are present in the pleura and have a large plasmablastic morphology.



(a) (b)

Figure 20.14 Burkitt lymphoma. (a) 'Starry sky' pattern due to the abundant histiocytes with apoptotic bodies; (b) Monotonous medium-sized cells with high proliferation.

have identified a distinctive profile of somatic mutations targeting the *ID3* (11%)/*TCF3* (40–70%) pathway, not detected in DLBCL, *DDX3X* (30–50%) and *CCND3* (38%).

High grade B-cell lymphomas (HGBL) with *MYC* and *BCL2* rearrangements

This category is recognised in both the ICC and WHO-HAEM5 and includes tumours with these two rearrangements. The ICC includes all tumours carrying these translocations independently of the morphology that may be DLBCL, intermediate between BL and DLBCL or blastoid. The WHO-HAEM5 splits tumours carrying the two translocations as DLBCL or HGBL depending on the morphology of the tumours cells. These tumours seem to derive from the dark zone of the germinal centre and express a mature B-cell phenotype with CD10 and BCL6 and have a very high proliferative index. Strong expression of BCL2 and MYC in a GCB large cell lymphoma should suggest the presence of the rearrangements of these genes that must be confirmed by genetic studies. Clinically, the patients are older than those with conventional BL, the disease is usually disseminated, and the bone marrow and peripheral blood may be involved. The clinical evolution is very aggressive, with poor response to conventional therapy. Some cases may represent transformed FL. However, independent of the possible biological significance, these patients should be recognised and studied separately from conventional DLBCL and BL. Cases with blastoid or intermediate morphology between BL and DLBCL but without this combination of rearrangements are diagnosed as high-grade B-cell lymphoma, NOS and probably is a more heterogeneous category. The ICC recommends to identify cases with similar morphologies carrying *MYC* and *BCL6* rearrangements as a separate category to allow further studies to better define these tumours whereas the WHO-HAEM5 recommends to include these cases as DLBCL, NOS.

Mediastinal grey zone lymphoma

These tumours are B-cell lymphomas that have clinical, morphological and/or immunophenotypic features that overlap those of PMBL and HL. The term mediastinal grey zone lymphomas (MGZL) is preferred for these tumours over the previous one of B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma. These cases are diagnosed frequently in young men presenting with a large mediastinal mass that is sometimes associated with surpraclavicular lymph nodes. Most of these cases are composed of sheets of large cells with a pleomorphic aspect, associated with some inflammatory infiltrate. The phenotype combines strong expression of a complete B-cell programme (CD20, CD79a, BOB-1, OCT2, PAX5) with CD30 and CD15 positivity. Mediastinal grey zone lymphomas have a similar mutational profile than PMBL and HL (*B2M*, *TNFAIP3*, *GNA13*). Tumours with overlapping features between Hodgkin and DLBCL outside the mediastinum have a mutational profile similar to DLBCL (Rearrangements of *BCL6* or *BCL2*, *TP53* mutations), and should be considered as DLBCL rather than included in this category. Composite lymphomas with clear areas of PMBL and HL are not included in this group and both components should be mentioned in the diagnosis. Microarray expression profiling has demonstrated the similarities between PMBL and HL and the intermediate profile of MGZL that probably represent evidence of plasticity of B cells.

Mature NK-cell/T-cell neoplasms

These tumours derive from natural killer (NK) cells and T cells of peripheral lymphoid organs and can be roughly subdivided into three groups: leukaemic, extranodal and nodal. The combination of morphological, phenotypic and molecular features is essential for the diagnosis of the different entities (Table 20.5). These are discussed in the following sections, with the exception of T-cell prolymphocytic leukaemia, T-cell large granular lymphocyte leukaemia and chronic lymphoproliferative disorders of NK cells, which are considered in Chapter 24.

Aggressive NK-cell leukaemia

This is a rare neoplasm, mainly observed amongst Asians, and exhibits systemic proliferation of NK cells almost always associated with EBV and an aggressive clinical course. Patients are usually middle-aged of either sex. The most commonly involved sites are the peripheral blood, bone marrow, liver and spleen (Figure 20.15). Fever and constitutional symptoms are common. Frequent complications are coagulopathy, haemophagocytic syndrome (HPS) or multiorgan failure. A specific overlap with extranodal NK/T-cell lymphoma may exist. Circulating

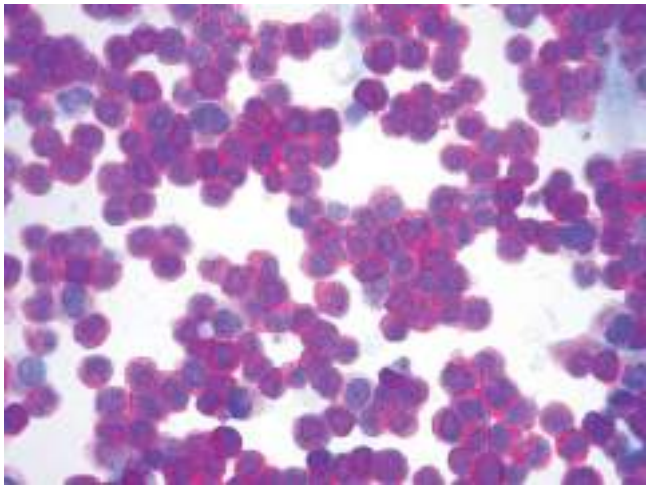


Figure 20.15 Cytospin from the peripheral blood of a patient with aggressive NK-cell leukaemia, stained for CD56 (alkaline phosphatase anti-alkaline phosphatase technique).

leukaemic cells exhibit a morphological spectrum, from 'normal LGL' to elements with atypical unfolded nuclei, open chromatin and distinct nucleoli. They express CD2, CD3ε, CD56, cytotoxic molecules and FASL. Surface CD3 is absent. T-cell receptor (*TCR*) genes are in germline configuration. Most cases pursue a fulminant clinical course.

EBV-positive T-cell and NK-cell lymphoproliferative disorders (LPDs) of childhood

They include: chronic active EBV (CAEBV) disease (T-cell and NK-cell phenotype) hydroa vacciniform LPD, classic, with cutaneous involvement and systemic mosquito bite allergy, and systemic EBV-positive T-cell lymphoma. They are prevalent in Far East Asia and people with native-American ancestry, with no sex predilection, and can develop shortly after primary EBV infection or in the setting of chronic active EBV infection. EBV-positive T-cell lymphoma of childhood has a fulminant course, usually associated with haemophagocytic syndrome. The other forms of T/NK LPDs show a broad range of clinical manifestations, from indolent, localised forms (such as hydroa vacciniform LPD and mosquito bite allergy) to systemic disease with fever, hepatosplenomegaly and lymphadenopathy, associated or not with cutaneous manifestations. Since an overlap of morphologic features is not infrequently observed amongst these EBV-positive LPDs of childhood, the knowledge of clinical data is of paramount importance for the correct diagnosis. On pathogenetic grounds, there is strong suggestion of a genetic defect in the host immune response to EBV. The infiltrating cells are usually small and lack significant cytological atypia, but at times are pleomorphic, medium/large with irregular nuclei and frequent mitoses (Figure 20.16). T-cell LPDs are positive for CD2, CD3, TIA and EBV, but negative for CD56, and have monoclonally rearranged TCR genes. NK-cell cases express CD2, CD3ε, CD56 and cytotoxic molecules, while lack surface CD3 and clonal rearrangement of *TCR* genes. Occasional cases with γδ-*TCR* and expression of *CCR4* have been reported.

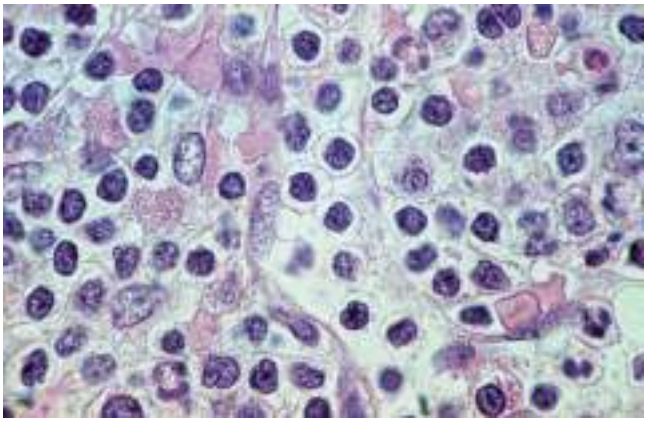


Figure 20.16 Bone-marrow involvement by EBV-positive lymphoproliferative disorder in a 16-year-old boy.

Adult T-cell leukaemia/lymphoma

Adult T-cell leukaemia/lymphoma (ATLL) is endemic in south-western Japan, the Caribbean basin and parts of Central Africa, its distribution resembling that of human T-cell leukaemia/lymphoma virus (HTLV)-I (see also Chapter 24). The disease has a long latency with exposure of affected individuals to the virus very early in life. Although causally linked to ATLL, HTLV-I itself is insufficient to cause neoplastic transformation, additional genetic events being required, such as mutations affecting *PLCG1*, *PRKCB*, *VAV1*, *IRF4*, *FYN*, *CARD11*, *STAT3* and *CCR4*, and intragenic deletions of *TP73*. Most patients present with widespread lymph node and peripheral blood involvement, the skin being affected in more than 50%. ATLL is characterised by variable clinical and histological features. In particular, the behaviour varies from acute (with usual hypercalcaemia and possible lytic bone lesions) to lymphomatous, chronic or smouldering, while the growth may consist of small- to large-sized pleomorphic cells or display anaplastic or even angioimmunoblastic-like morphology (Figure 20.17). Tumour elements are usually $CD2^+CD3^+CD5^+CD25^+CD7^-$. Most cases are $CD4^+CD8^-$, a few $CD4^-CD8^+$ or $CD4^+CD8^+$. CCR4 and FoxP3 are frequently expressed, while cytotoxic molecules are absent. CD30 positivity is at times detected, especially in cases lacking FoxP3, by heralding a more aggressive clinical course. CCR4 and CD30 can represent the targets for *ad hoc* therapy with monoclonal antibodies. *TCR* genes are clonally rearranged. Clinical subtypes, age, performance status, serum calcium and lactate dehydrogenase levels are major prognostic indicators. The acute and lymphomatous variants have a short survival (2 weeks to more than 1 year), while the chronic and smouldering

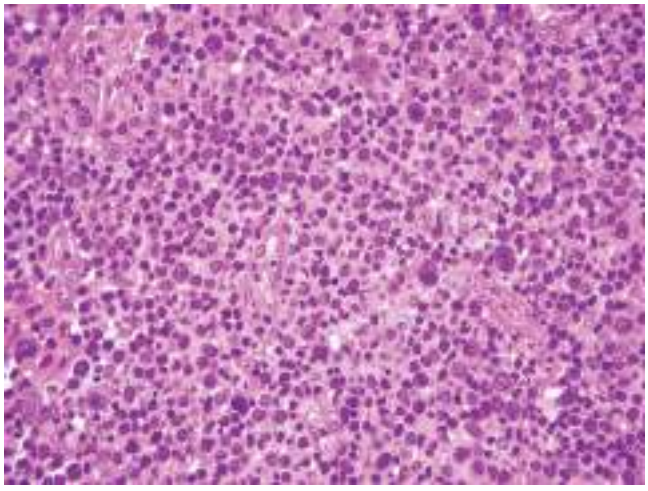


Figure 20.17 Lymph node infiltration in a patient with adult T-cell leukaemia/lymphoma.

forms behave more indolently, but can progress to an acute phase (see also Chapter 24).

Extranodal NK/T-cell lymphoma, nasal type

Extranodal NK/T-cell lymphoma, nasal type, more often occurs in adult males from Far East Asia, and from native-American ancestry (see also Chapter 24). The tumour is EBV positive with type II latency, although LMP-1 expression is variable. Usually, these lymphomas affect the nasal cavity, nasopharynx, paranasal sinuses and palate. Extranasal locations include the skin, soft tissue, gastrointestinal tract and testis. Patients with nasal disease suffer from nasal obstruction or epistaxis, or extensive mid-facial destruction (so-called lethal midline granuloma). Irrespective of the site of presentation, the lymphomatous infiltrate is diffuse with angiocentric and angiodestructive features and frequent necrosis. Neoplastic cells may be small, medium-sized, large or anaplastic with numerous mitotic figures and azurophilic granules in Giemsa-stained touch preparations (Figure 20.18). Phenotypically, they are positive for CD2, CD56, CD3ε and cytotoxic markers, but negative for surface CD3. About one-third of cases expresses CD30. *TCR* and *IGHV* genes are usually in germline configuration, the former being clonally rearranged in a few instances. The prognosis is variable, being always poor for cases occurring outside the nasal cavity. Survival has been improved with more intensive regimens, including up-front radiotherapy and L-asparaginase. The WHO-HAEM5 recognises these tumours but has eliminated the term nasal type.

Intestinal T-cell lymphomas

Intestinal T-cell lymphomas include enteropathy-associated T-cell lymphoma (EATL), monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL, intestinal T-cell lymphoma, NOS and indolent clonal T-cell lymphoproliferative disorder of the gastrointestinal tract, and Indolent NK-cell lymphoproliferative disorder of the gastrointestinal tract (see also Chapter 24).

Enteropathy-associated T-cell lymphoma

EATL is an intestinal tumour of intraepithelial T lymphocytes that occurs in individuals with coeliac disease. It consists of medium-sized to large lymphoid cells admixed with numerous histiocytes and eosinophils and associated with necrosis. The adjacent small intestinal mucosa shows villous atrophy, crypt hyperplasia, increased lamina propria lymphocytes and plasma cells and intraepithelial lymphocytosis (Figure 20.19). The disease occurs more often in areas with a high prevalence of coeliac disease (i.e. northern Europe) and has a strong association with *HLA-DQA1*0501* and *HLA-DQB1*0201* genotypes. EATL may be preceded by type II refractory coeliac disease that is now included in the ICC classification. In particular, 30–52% of patients with type II refractory coeliac disease (RCD2), characterised by an aberrant clonal intraepithelial population, evolve to frank EATL within 4–6 years. Patients (usually adults) present with abdominal pain, frequently associated with intestinal perforation. The tumour forms one or more ulcerating mucosal masses that invade the wall of the intestine (frequently jejunum or ileum). Neoplastic cells are positive for CD3, CD7, CD103, TCR-β and cytotoxic molecules, negative for CD4, CD5 and MATK, with variable expression of CD8 and CD30. TCRβ and γ genes are always clonally rearranged. Cytogenetics reveals that most EATLs (~80%) either display gains of the 9q34 region, which harbours known proto-oncogenes (e.g. *NOTCH1*, *ABL1* and *VAV2*) or, alternatively, deletions of 16q12.1. Similar changes are also observed in MEITL. However, EATL frequently shows gains of chromosomes 1q and 5q, which are less common in MEITL. Loss at 9p21 (*CDKN2A/B*) and 17p12-13.2 (*TP53*) are common (25–50%) Sequencing studies have shown recurrent mutations in constituents of the JAK-STAT signalling pathway. The prognosis is poor, with death frequently resulting from abdominal complications.

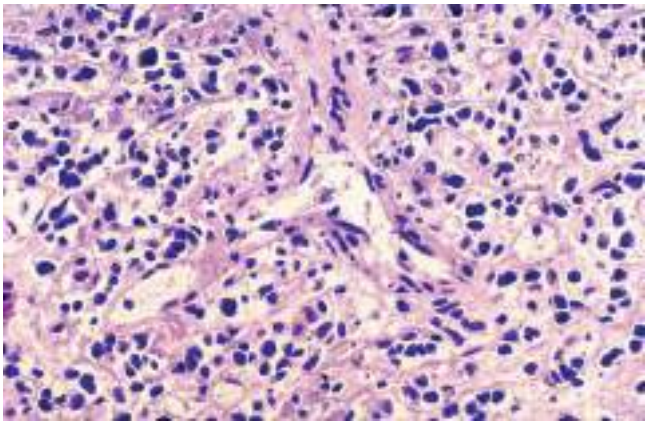


Figure 20.18 Destructive lesion of the nasal cavities in the course of extranodal NK/T-cell lymphoma, nasal type.

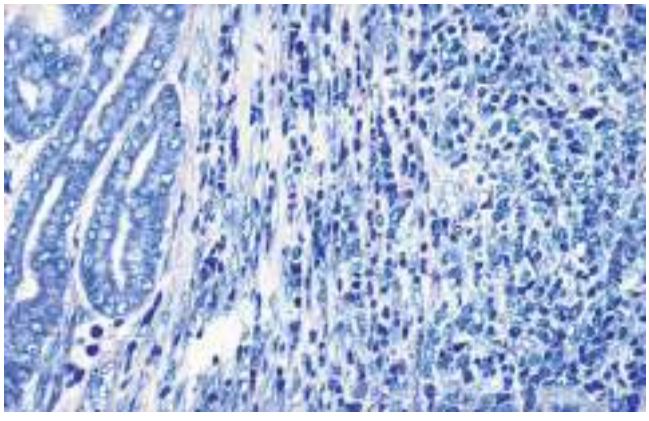


Figure 20.19 Enteropathy-associated T-cell lymphoma. Remnants of the mucosa are seen on the left-hand side.

Monomoprphic intestinal T-cell lymphoma

MEITL is a primary intestinal T-cell lymphoma derived from intraepithelial lymphocytes. It has no clear association with coeliac disease. MEITL has a worldwide distribution, with a higher prevalence amongst Asians, Hispanics and American indigenous populations. It is composed of monomorphic medium-sized cells with no inflammatory background and rare necrosis. Enteropathic changes in the surrounding or adjacent mucosa consists of focal or extensive epitheliotropism in the absence of villous atrophy and crypt hyperplasia. 'MEITL has a distinctive immunophenotype ($CD3^+CD4^-CD5^-CD8^+CD56^+$, $MATK^+$). In a significant proportion of cases, neoplastic cells carry γδ-TCR. Gain of 9.34.3 and 8q24 (*MYC*) are detected in most instances. Activating mutations of *STAT5B* are frequently observed, along with *SETD2* mutations. EBV is negative.

Intestinal T-cell lymphoma, NOS

Intestinal T-cell lymphoma, NOS corresponds to tumours more often localised in the colon, which cannot be assigned to any of the above categories. This can often be due to an inadequate biopsy and/or incomplete clinical information.

Indolent clonal T-cell and NK lymphoproliferative disorders of the gastrointestinal tract

Indolent clonal T-cell lymphoproliferative disorder of the gastrointestinal tract is a clonal T-cell disorder that can affect the mucosa of the gastrointestinal tract, most commonly of the small intestine and colon. The lymphoid proliferation appears monotonous, consists of small cells and involves the lamina propria, usually sparing the mucosal glands. It expresses CD3, mainly CD4 and less frequently CD8 or double negative. The Ki-67 marking is below 10%. The clinical course is indolent and most patients do not respond to conventional chemotherapy. Occasional cases progress to a higher-grade T-cell lymphoma with spread beyond the gastrointestinal tract. The NK lymphoproliferative disorder of the gastrointestinal tract is now recognised as a neoplasm with very indolent behaviour.

Hepatosplenic T-cell lymphoma

Hepatosplenic T-cell lymphoma (HSTCL) is a rare neoplasm derived from cytotoxic T cells, mostly of the γδ type. It mainly affects young adults, with male predominance. Up to 20% of these lymphomas arise in the setting of chronic immunosuppression, usually for solid organ transplantation or prolonged antigenic stimulation. It may also occur in patients, especially children, treated with azathioprine and infliximab for Crohn's disease. Clinically, it is characterised by hepatosplenomegaly, systemic symptoms and marked thrombocytopenia. Morphologically, neoplastic cells are monotonous, with medium-sized nuclei, loosely condensed nuclear chromatin, inconspicuous nucleoli and a rim of pale cytoplasm. In the spleen, they infiltrate the red pulp cords and sinuses with atrophy of the white pulp, while in the liver and bone marrow, they show intrasinusoidal diffusion (Figure 20.20). Phenotypically, most cases are positive for CD3 and TCR-δ1, partly positive for CD56 and CD8, and negative for TCR-αβ, CD4 and CD5 (Table 20.5). A minority carries the α/β dimer. The cells express TIA1 and granzyme-M but not granzyme-B and perforin. They have rearranged *TCR*-γ genes. Cases of γδ origin show biallelic rearrangement of *TCR*-δ genes. Isochromosome 7q is present in most instances. Trisomy 8 and loss of a sex chromosome may also be present. In more than 60% of cases, chromatin-modifying genes, including *SETD2*, *INO80* and *ARID1B,* are mutated. Furthermore, in 40% of HSTCLs, missense mutations in *STAT5B* and more rarely *STAT3* have been found, in keeping with the enrichment in genes of the JAK/STAT pathway of gene expression profile. The course is aggressive, with initial response to chemotherapy, but rapid relapse. The median survival is less than 2 years.

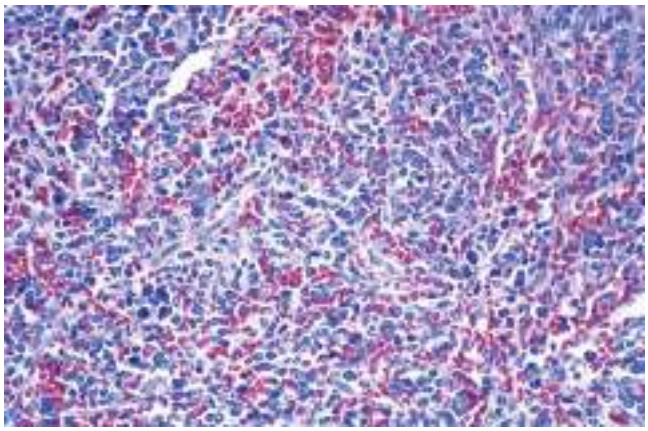


Figure 20.20 Splenic red pulp involvement by hepatosplenic T-cell lymphoma.

Subcutaneous panniculitis-like T-cell lymphoma

Subcutaneous panniculitis-like T-cell lymphoma is a rare cytotoxic T-cell lymphoma that preferentially infiltrates subcutaneous tissue, is slightly commoner in females and has a broad age spectrum. Cases expressing *TCR*-γδ are excluded and instead classified as primary cutaneous γδ T-cell lymphoma. About 20% of patients have an associated autoimmune disease, mostly systemic lupus erythematosus. Patients present with multiple subcutaneous nodules, usually in the absence of other involved sites. Systemic symptoms are recorded in more than 50% of patients. Cytopenias and elevated liver function tests are common, and frank HPS is seen in 15–20%. The infiltrate consists of cells homogeneously sized in the single case, although the cell size can vary form case to case. It involves the fat lobules with riming around individual fat cells and usually

Table 20.5 Immunophenotypic features of common T-cell neoplasms.

Neoplasm	CD3	CD5	CD7	CD4	CD8	CD30	TFH markers	*TCR*	CD56	Cytotoxic granules	EBV
T-PLL	+	–	+	+/–	–/+	–	–	αβ	–	–	–
T-LGL	+	–	+	–	+	–	–	αβ	–	+	–
NK-LGL	–	–	+	–	+/–	–	–	–	+	+	+
Extranodal NK/T-cell lymphoma	–; +	–	–/+	–	–	–	–	–	+	+	++
Hepatosplenic T-cell lymphoma	+	–	+	–	–	–	–	γδ>αβ	+/–	+	–
Enteropathy-type T-cell lymphoma	+	+	+	–	+/–	+/–	–	αβ>>γδ	–	+	–
Mycosis fungoides	+	+	–/+	+	–	–/+	–	αβ	–	–	–
Subcutaneous panniculitis like T-cell lymphoma	+	+/–	+/–	–	+	–	–	αβ	–	+	–
Primary cutaneous γδ T-cell lymphoma	+	–	+	–	–	–	–	γδ	+	+	–
PTCL-NOS	+/–	+/–	+/–	+/–	–/+	–/+	–	αβ>γδ	–/+	–/+	–/+
Angioimmunoblastic T-cell lymphoma	+	+	+	+/–	–/+	–	+	αβ	–		+/–
ALCL	+/–	+/–	+/–	–/+	–/+	++	–	αβ	–	+	–

Cytotoxic granules; TIA-1, perforin and granzyme; TCR: T-cell receptor gene; TFH (T follicular helper cell) markers CXCL13, ICOS, BCL6, CD10, PD1.

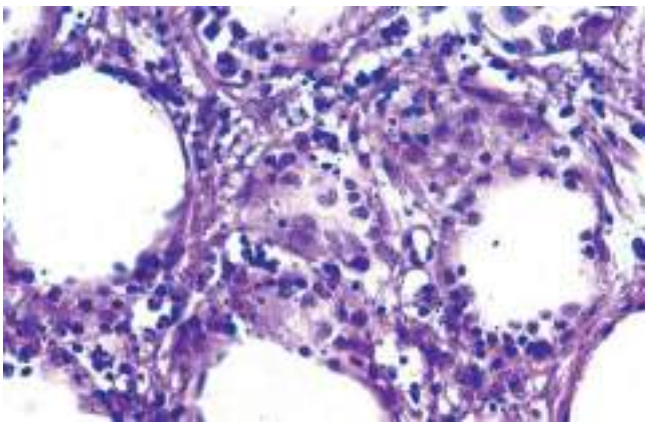


Figure 20.21 Example of subcutaneous panniculitis-like T-cell lymphoma. Features of 'rimming' are easily seen.

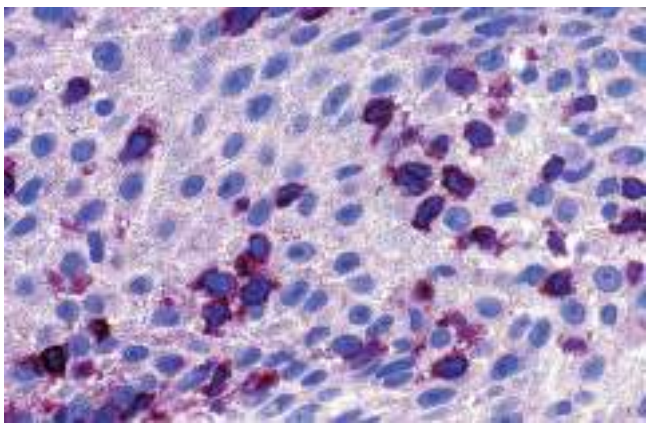


Figure 20.22 Mycosis fungoides. The epidermis is infiltrated by neoplastic T cells with cerebriform nuclei and strong CD3 expression.

spares septa as well as the overlying dermis and epidermis (Figure 20.21). Vascular invasion is seen occasionally, while necrosis and karyorrhexis are common. The cells have a mature αβ T-cell phenotype (βF1$^+$), usually CD8$^+$ with expression of cytotoxic molecules and negative for CD56. The neoplastic cells show rearrangement of TCR genes and are negative for EBV. Dissemination to lymph nodes and other organs is rare. The 5-year overall survival (OS) is 80%; however, if HPS occurs, the prognosis is poor.

Mycosis fungoides

Mycosis fungoides is the commonest primary cutaneous T-cell lymphoma (CTCL) and is characterised by epidermotropic infiltrates of small to medium-sized T lymphocytes with cerebriform nuclei (Figure 20.22) (see also Chapter 24). It usually affects adults or the elderly with a male predominance, but can also occur in children and adolescents. The disease is limited to the skin, often confined to sun-protected areas, with classical evolution of

patches, plaques and tumours. Extracutaneous dissemination occurs in advanced stages. Erythrodermic manifestations at presentation or during the course of the disease are rare. They lack the haematological criteria of Sézary syndrome. Mycosis fungoides slowly progresses over years or sometimes decades. Histological transformation (i.e. >25% blasts in the dermal infiltrates) occurs mainly in the tumour stages. The typical phenotype is $CD2^+CD3^+TCR\text{-}\beta^+CD5^+CD4^+CD8^-CD7^-$. Recently, some reports have highlighted occasional cases expressing follicular T-helper (FTH) markers or γδ T-cell phenotype. Partial expression of CD30 may be found in all stages but is more frequent in plaques and tumours. *TCR* genes are clonally rearranged in most patients, when sensitive techniques are applied. By whole-genome sequencing, it has been observed that *PD1* mutations drive aggressive behaviour. The single most important prognostic factor is the clinical stage. In the more advanced stages, the prognosis is poor, as it is in the case of histological transformation. Rare clinical–morphological variants include folliculotropic mycosis fungoides, pagetoid reticulosis and granulomatous slack skin.

Sézary syndrome

Sézary syndrome (SS) is a distinct entity, which is defined by the triad of erythroderma, generalised lymphadenopathy and presence of neoplastic T cells with cerebriform nuclei (so-called Sézary cells) in skin, lymph nodes and blood (see also Chapter 24). In addition, one or more of the following criteria are required: an absolute Sézary cell count ≥1000/μl, a CD4/CD8 ratio over than 10/1 and loss of one or more T-cell antigens. SS is a rare disease accounting for less than 5% of CTCLs. It occurs in adults, with a male predominance. The histological and phenotypic features in Sézary syndrome may be similar to those of mycosis fungoides. *TCR* genes are clonally rearranged. NGS studies have revealed mutations predicted to affect chromatin, immune surveillance, MAPK signalling, NF-kB signalling, PI-3-kinase signalling, RHOA/cytoskeleton remodelling, RNA splicing), T-cell receptor signalling and T-cell differentiation. Mutations or deletions of *TP53* and deletions of *CDKN2A* are frequently encountered. The 5-year OS is 10–30%.

Primary cutaneous CD30-positive T-cell lymphoproliferative disorders

These lesions include primary cutaneous anaplastic large-cell lymphoma (C-ALCL) and lymphomatoid papulosis, which form a spectrum with overlapping histopathological and phenotypic features. Their clinical appearance and course are therefore critical for the diagnosis.

Primary cutaneous anaplastic large-cell lymphoma

C-ALCL is composed of large cells with an anaplastic, pleomorphic or immunoblastic morphology, the vast majority of which express CD30. By definition, C-ALCL should not be preceded by or coincide with mycosis fungoides. It must also be distinguished from systemic ALCL, which is a different entity. Most patients present with solitary or localised nodules or tumours, and sometimes papules located on the trunk, face, extremities or buttocks that often undergo ulceration and sometimes show partial or complete spontaneous regression, as in lymphomatoid papulosis. Besides CD30 positivity, the neoplastic cells display an activated $CD4^+$ T-cell phenotype, with variable loss of CD2, CD5 and/or CD3. Unlike systemic ALCL, C-ALCL expresses CLA, but not EMA or ALK. The latter molecule has been reported in some exceptional cases, which run the same course as the negative ones. Of course, under these circumstances, it is pivotal to exclude a systemic ALCL ALK^+. Unlike Hodgkin and Reed–Sternberg cells, staining for CD15 is generally negative. Most cases show clonal *TCR* gene rearrangement. In a proportion of cases, it has recently found the occurrence of *DUSP22* rearrangement, which requires the exclusion of an underlying systemic ALCL, ALK^- (see below). The prognosis is favourable, with a 10-year OS of approximately 90%, although local relapses are frequent.

Lymphomatoid papulosis

Lymphomatoid papulosis (LyP) is a chronic, recurrent, self-healing skin disease that most often occurs in adults, with male preponderance, affects trunk and extremities and is characterised by paradoxical eruptions of papular, papulonecrotic and/or nodular lesions that tend to disappear within 3–12 weeks, possibly leaving a scar. The duration of the disease may vary from several months to over 40 years. The prognosis is excellent. Morphologically, the large CD30-positive cells may be intermingled with a variable number of inflammatory cells or predominate as a monotonous population. The phenotype is similar to that of C-ALCL. Depending on the amount and distribution of atypical cells, five variants of LyP (A–E) are recognised, which can mimic other conditions, including mycosis fungoides and primary cutaneous CD8-positive aggressive epidermotropic cytotoxic T-cell lymphoma. More recently, a further variant has been reported, characterised by *DUSP22* rearrangement, as observed in ALCL ALK^- (see below). Clonally rearranged *TCR* genes are detected in about 60% of lesions.

Primary cutaneous peripheral T-cell lymphoma, rare types

Primary cutaneous γδ T-cell lymphoma

This tumour consists of a clonal proliferation of activated γδ T cells with a cytotoxic phenotype. This lymphoma is rare (1% of CTCLs) and more often observed in the extremities of adults with no sex predilection. It may be predominantly epidermotropic with patches/plaques or may give rise to deep dermal or subcutaneous tumours, with or without epidermal necrosis and ulceration. Dissemination to mucosal and other extranodal sites

is frequent. HPS may occur. 'B' symptoms are seen in most patients. The tumour cells characteristically exhibit a βF1−CD3$^+$CD2$^+$CD5$^-$CD7$^{+/-}$CD56$^+$ phenotype with strong expression of cytotoxic proteins. The cells are strongly positive for *TCR*-δ and γ with appropriate detection methods. The cells show clonal rearrangement of the *TCR*-γ and *TCR*-δ genes. *TCR*-β may be rearranged or deleted but is not expressed. Primary cutaneous γδ T-cell lymphoma is resistant to multiagent chemotherapy and/or radiation and has a poor prognosis, with a median survival of approximately 30 months.

Primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma

Primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma is characterised by proliferation of epidermotropic CD8$^+$ cytotoxic T cells, aggressive clinical behaviour and a short survival. It should be differentiated from LyP variant E and CD8$^+$ mycosis fungoides. Clinical information is essential. Conversely to the latter forms, primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma shows rapid dissemination of ulcerated nodules, tumours or plaques, with possible spread to lungs, testes, CNS and oral mucosa.

Primary cutaneous acral CD8$^+$ T-cell lymphoproliferative disorder

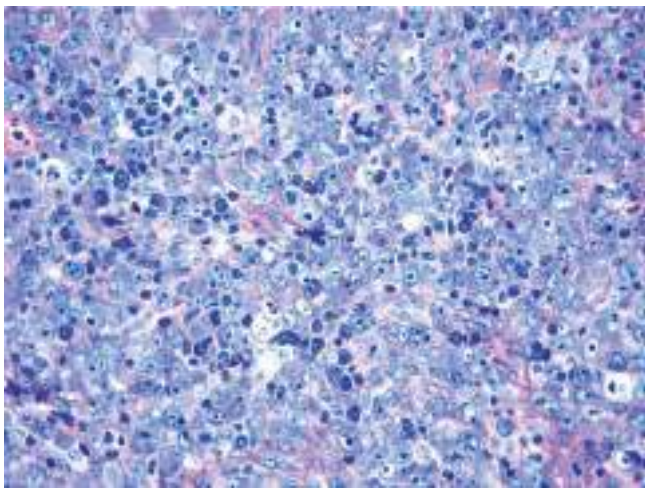
This is a rare cutaneous tumour characterised by skin infiltration of clonal atypical medium-sized to large cytotoxic lymphocytes CD3$^+$, CD8$^+$, βF1$^+$ and TIA1$^+$, but granzyme$^-$ and perforin$^-$. The Ki-67 index is below 10%. The tumour is clinically characterised by preferential involvement of acral sites (in particular the ears) and by a good prognosis. Both the ICC and WHO-HAEM5 have proposed to substitute the previous term of lymphoma by lymphoproliferative disorder to emphasise the very indolent nature of these tumours.

Primary cutaneous CD4$^+$ small/medium T-cell lymphoproliferative disorder

Primary cutaneous CD4$^+$ small/medium T-cell lymphoproliferative presents with a solitary skin lesion in face, neck or upper trunk. The atypical CD4$^+$ T cells are admixed with variable amounts of small reactive CD8$^+$ T-cells, B-cells, plasma cells and histiocytes (including multinucleated giant cells). Patients have an excellent prognosis, being treated with intralesional steroids, surgical excision or radiotherapy. Local recurrences are rare.

Peripheral T-cell lymphoma, not otherwise specified

Peripheral T-cell lymphoma not otherwise specified (PTCL, NOS) is a heterogeneous group of tumours whereby the diagnosis is established by exclusion of other T-cell lymphoma entities (see also Chapter 24). It accounts for approximately 30% of PTCLs in Western countries. Most patients are adults, with a slight male predominance, and show peripheral lymphadenopathy and 'B' symptoms. Generalised disease is often encountered, with bone marrow, liver, spleen and extranodal tissue involvement. Leukaemic spread is uncommon. The cytological spectrum is extremely broad, from highly polymorphous to monotonous. Clear cells and Reed–Sternberg-like elements can be seen. An inflammatory background is often present (Figure 20.23). The differential diagnosis with ALCL ALK$^-$ may at times be difficult. In fact, some PTCL, NOS tumours almost exclusively consist of CD30$^+$ elements, although they lack hallmark cells and cytotoxic profile. PTCL/NOS usually displays an aberrant T-cell phenotype, with frequent loss of CD5 and CD7. A CD4$^+$CD8$^-$ profile predominates



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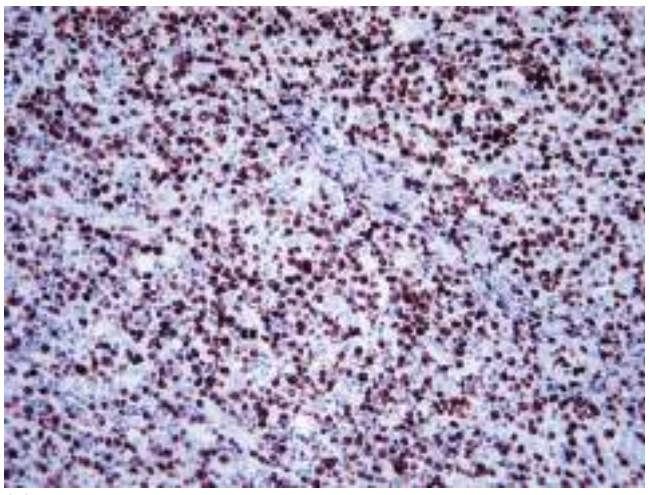


(b)

Figure 20.23 Peripheral T-cell lymphoma not otherwise specified. (a) The tumour consists of large cells with immunoblastic-like appearance. (b) The proliferative rate as shown by Ki-67 marking is very high.

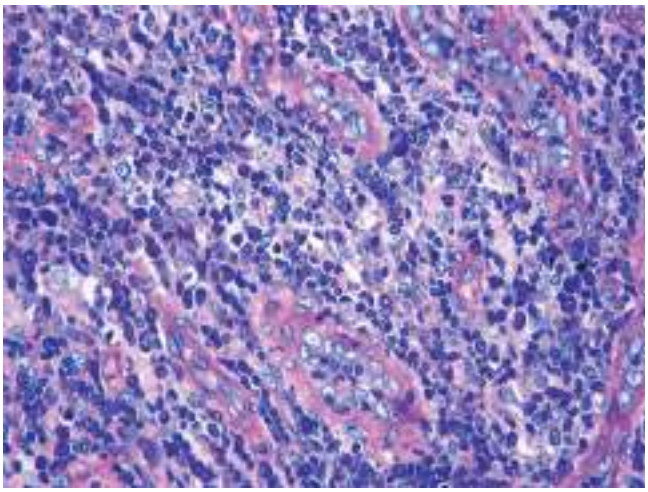
in nodal cases. $CD4^+CD8^+$ or $CD4^-CD8^-$ is sometimes seen, as is CD8, CD56 and/or cytotoxic granule expression (Table 20.5). Aberrant CD20 and/or CD79a expression is occasionally encountered. In half of cases, 25–100% of lymphomatous elements carry CD30, which can represent a therapeutic target. Proliferation is high and Ki-67 rates over 80% are associated with poor outcome. *TCR* genes are clonally rearranged. EBV integration is found in some cases. Gene and miRNA expression profiling studies have revealed signatures distinct from those of other PTCLs. In addition, they have allowed the identification of morphologically unrecognisable subtypes, respectively related to T_H1, T_H2 and cytotoxic T-cells. In particular, three main subgroups have been reported, respectively provided with a *TBX21*, *GATA3* or null gene signature. This signature has recently become applicable to formalin-fixed, paraffin-embedded tissue sections, by an immunohistochemical algorithm. The *TBX21*, *GATA3* and null cases are characterised by a more favourable, aggressive and intermediate clinical course, respectively. The behaviour of *TBX21* cases becomes even better, if cytotoxic cases are excluded from their context, which are provided with the worst prognosis amongst PTCLs/NOS. NGS studies have revealed mutations of *TET2, DNMT3A, KMT2D, KMT2C, SETD2*, *LATS1, STK3, ATM, TP53* and *TP63*. PTCL/NOS is highly aggressive with poor response to chemotherapy, frequent relapses and 5-year OS of 20–30%, unless autologous bone-marrow transplantation is used in the first line.

Follicular helper T-cell lymphoma

Both classifications have proposed a unifying view of peripheral T-cell lymphomas with a T follicular helper (TFH) phenotype. Angioimmunoblastic T-cell lymphoma (AITL) type represents the commonest form of TFH lymphomas. Collectively, these neoplasms – which account for at least 25% of PTCLs – express at least two, preferably three of the markers carried by normal FTH cells, i.e. CD10, BCL6, CXCL13, PD1, SAP, ICOS and CCR5 (Figure 20.24). AITL is characterised by a polymorphous infiltrate constantly involving lymph nodes with prominent proliferation of high endothelial venules and follicular dendritic cells (Figure 20.24). Spleen, liver, skin and bone marrow are also frequently affected. It occurs in middle-aged/elderly individuals of both sexes. Virtually all patients present with advanced-stage disease, systemic symptoms, polyclonal hypergammaglobulinaemia, circulating immune complexes, cold agglutinins with haemolytic anaemia, positive rheumatoid factor and anti-smooth-muscle antibodies. Skin rash is frequently observed. Other common findings are pleural effusion, arthritis and ascites. AITL is characterised by partial effacement of the lymph node architecture with sparing of the peripheral cortical sinuses and some follicles. The neoplastic infiltrate is composed of small- to medium-sized lymphocytes, with clear to pale cytoplasm and mild cytological atypia. They often represent a minority of the examined population, being admixed with small reactive lymphocytes, eosinophils, plasma cells and histiocytes. An expansion of EBV-positive B immunoblasts is usually present. TCR genes are clonally rearranged in most cases. Clonal IGHV gene rearrangements are found in about 25–30% of cases and correlate with expanded EBV-positive B cells that may form a large B-cell lymphoma. NGS has identified mutations in *RHOA*, *TET2*, *IDH2*, *DNM3TA*, *FYN* and *ITK/SYK* genes. The search for *RHOA* and *IDH2* mutations may assist in proving the neoplastic nature of the process in cases with a small number of neoplastic cells, in which the detection of *TCR* rearrangement may be hampered. Notably the same clinical and cytological features as well as GEP and WES findings have also been encountered in tumours that in the past were classified as PTCL/NOS and now are named TFH lymphoma, NOS both in the ICC and WHO-HAEM5. These have a diffuse growth pattern without overt hyperplasia of FDCs and HEVs. A third type of TFH lymphomas is recognised with a predominantly follicular growth pattern named TFH lymphoma, follicular type. The clinical course of AILD and other nodal PTCLs of TFH derivation is aggressive with a median survival of less than 3 years. Patients often succumb to infectious complications. Supervening large B-cell lymphoma (often but not invariably EBV positive) can occur.

Anaplastic large-cell lymphoma, ALK^+

ALCL ALK^+ is characterised by large neoplastic cells with abundant cytoplasm and kidney/horseshoe-shaped nuclei (so-called hallmark cells), with a translocation involving the *ALK* gene and expression of both ALK protein and CD30. ALCL with similar morphology and phenotype, but lacking *ALK* rearrangement and ALK protein, is considered as a separate category (ALCL ALK^-). ALCL ALK^+ shows a higher prevalence in the first three decades of life and a male predominance, and frequently involves both lymph nodes and extranodal sites. Most patients present with stage III–IV disease and 'B' symptoms. The morphology may be variable. The 'common' appearance (60% of cases) consists of large tumour cells, frequently with hallmark appearance, that tend to grow within the sinuses, resembling a metastatic tumour (Figure 20.25). Some cases may have a lymphohistiocytic pattern (10%) characterised by few tumour cells overwhelmed by reactive histiocytes. A small-cell variant (5–10%) has been recognised that seems to have a worse prognosis. Some cases (3%) may mimic HL, but the cells are ALK-positive and CD15-negative. Relapses may reveal morphological features different from those seen initially. The tumour cells are CD30 positive. In cases with t(2;5)/*NPM–ALK* translocation, ALK staining is both cytoplasmic and nuclear. In cases with variant translocations, ALK staining may be membranous or cytoplasmic. Most ALCL ALK^+ are positive for EMA, express cytotoxic markers and bear one or more T-cell antigens, although some may have



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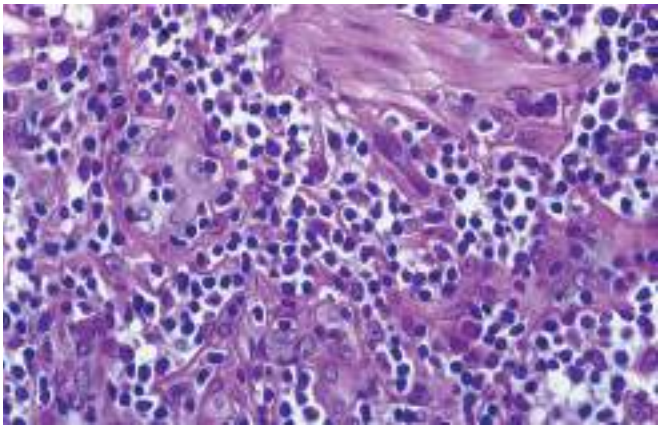

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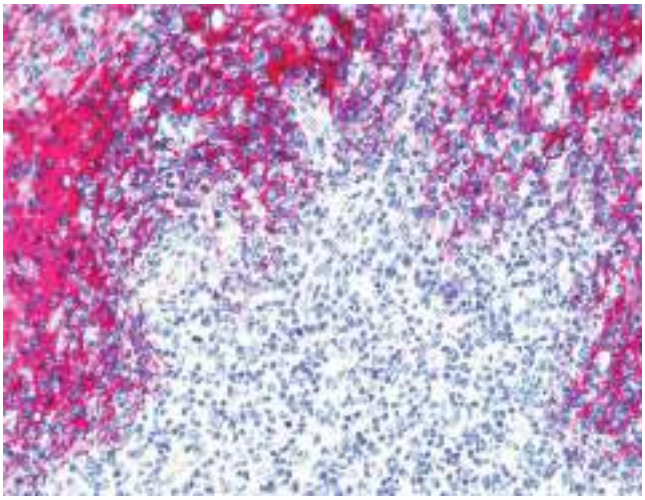

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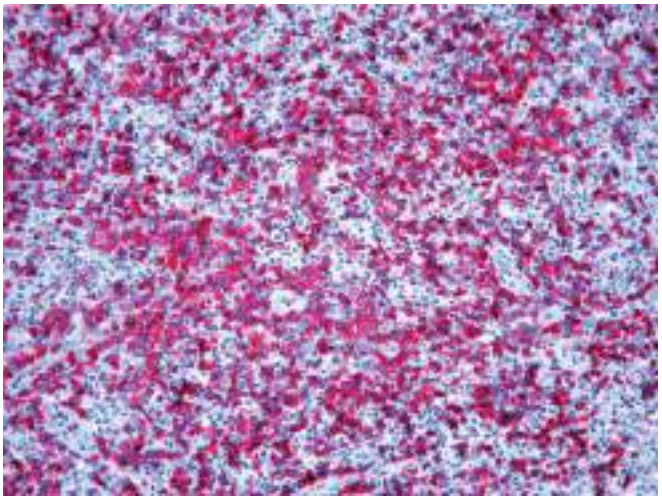

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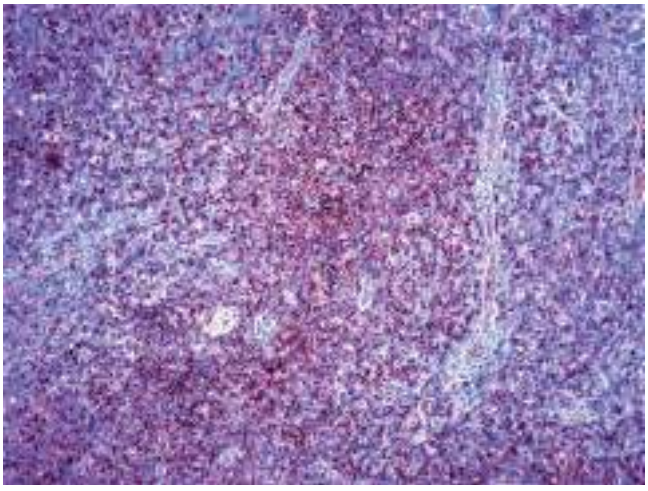

(e)

Figure 20.24 Angioimmunoblastic T-cell lymphoma. (a) Low-power view: note the amount of high endothelial venules as well as the cellular pleomorphism; (b) At higher magnification, neoplastic cells display irregular nuclear contours and a rim of clear cytoplasm; (c) Hyperplasia of follicular dendritic cells recognised with CD21 staining. The tumour cells are positive for CD10 (d) and CXCL13 (e).

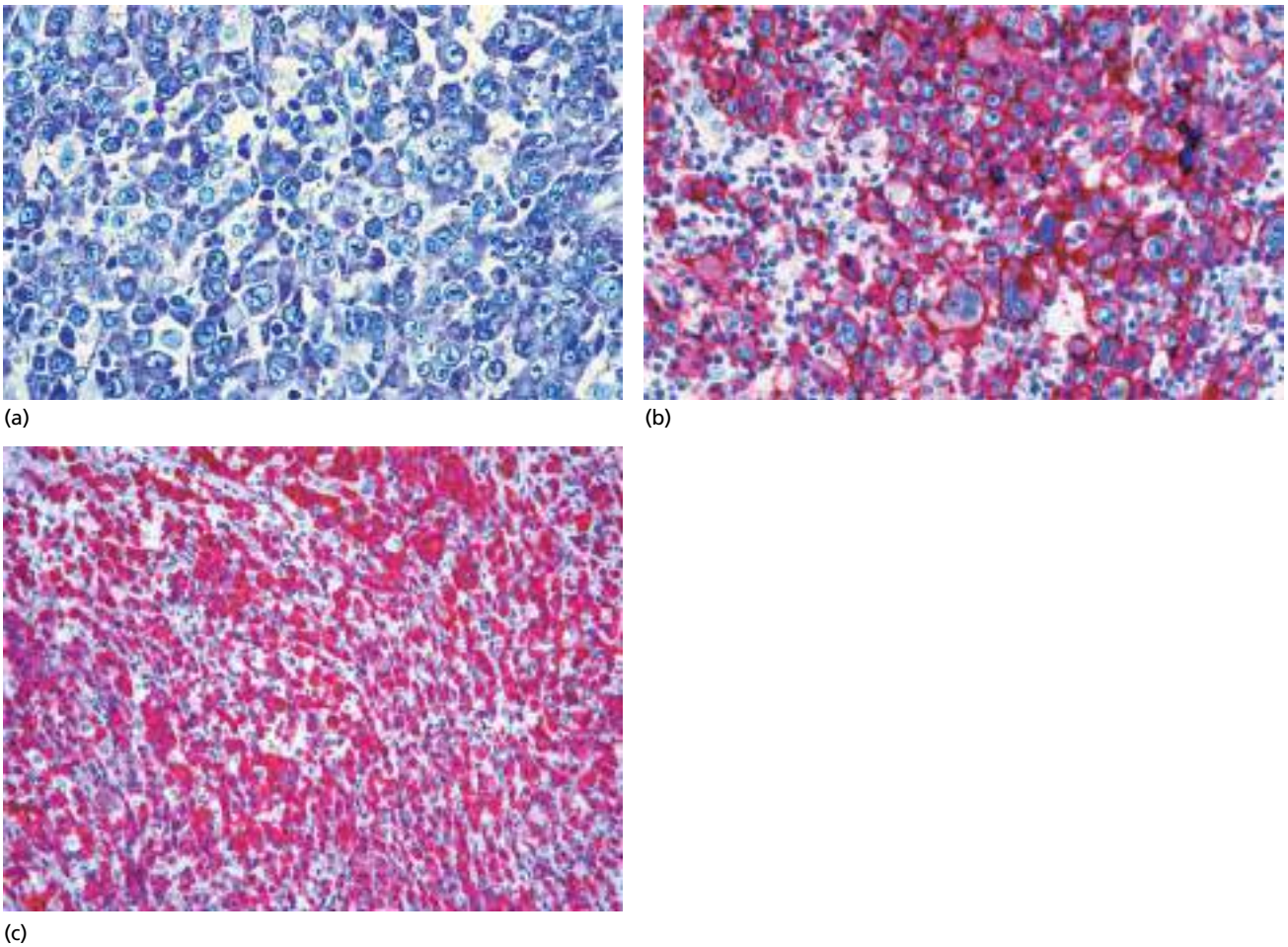

Figure 20.25 Anaplastic large-cell lymphoma, ALK⁺. (a) Some hallmark cells can be easily seen. (b, c) Expression of CD30; (b) and ALK protein (c) (alkaline phosphatase anti-alkaline phosphatase, Gill's counterstain, ×500 and ×250, respectively).

null phenotype. EBV is negative and the *TCR* gene is rearranged. The most frequent genetic alteration is t(2;5)(p23;q35) involving the *ALK* and nucleophosmin (*NPM*) genes. Variant translocations lead to fusion of the *ALK* gene with other partners on chromosomes 1, 2, 3, 17, 19, 22 and X. All these aberrations result in upregulation and different subcellular distribution of ALK protein, but do not have clinical relevance. ALCL ALK⁺, ALCL ALK⁻ and PTCL/NOS have different chromosomal and gene expression profiles. The IPI predicts the clinical outcome and the 5-year OS is about 80%.

Anaplastic large-cell lymphoma, ALK⁻

ALCL ALK⁻ is morphologically and phenotypically indistinguishable from 'common' ALCL ALK⁺ (Figure 20.26). The tumour affects adults, with a slight male predominance, involves lymph nodes and, less frequently, extranodal sites. Most patients are stage III–IV, often with 'B' symptoms. Usually, the organ architecture is effaced by solid cohesive sheets of neoplastic cells. In the lymph node, the latter tends to diffuse through sinuses, mimicking metastatic carcinoma. Most cases show clonal *TCR* gene rearrangement and EBV is negative. The main differential diagnosis is with PTCL, NOS and classical HL. In this regard, PAX5 staining is useful: classical HL shows weak positivity in most cases, a finding not observed in ALCL ALK⁻. In contrast, the distinction between ALCL ALK⁻ and CD30⁺ PTCL/NOS is not always easy on conventional grounds, especially when no careful search for hallmark cells and cytotoxic markers is performed. Molecular tools based on gene expression and miRNA profiling can assist in this differential diagnosis, even when applied to formalin-fixed, paraffin-embedded (FFPE) material. Such distinction may be relevant. In fact, on average ALCL ALK⁻, although more aggressive than ALCL ALK⁺ (5-year OS 49% versus 80%) has a more favourable course than CD30⁺ PTCL/NOS (5-year OS 32 versus 49%). Recent molecular studies have shed new light on the pathobiology of ALCL ALK⁻. First, a proportion of cases carry *JAK1* and *STAT3* mutations that cause downstream the same effects as the t(2;5)

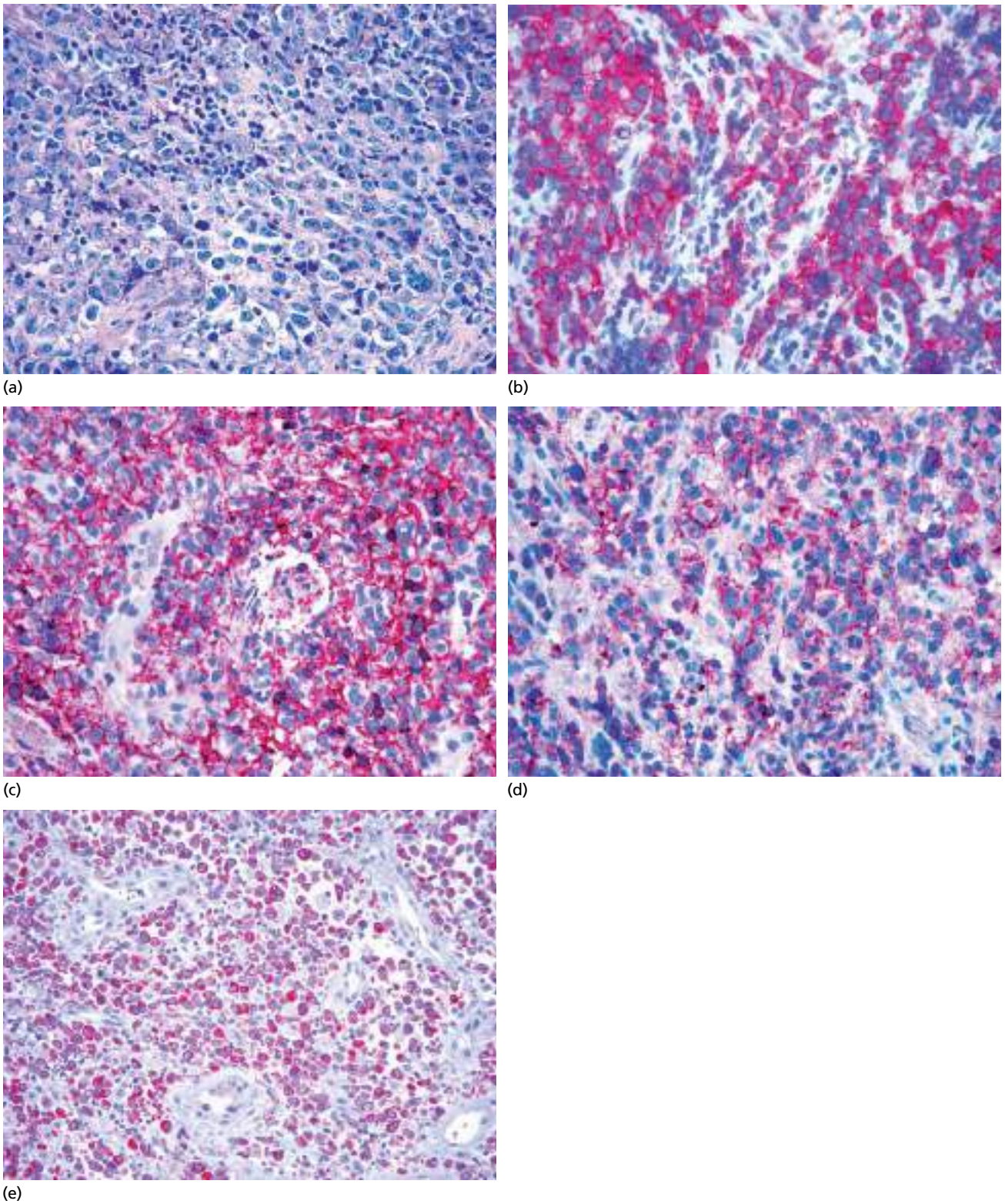

Figure 20.26 Anaplastic large-cell lymphoma, ALK$^-$. (a) The tumour consists of large cells partly showing hallmark appearance, and strong positivity for CD30 (b), CD45 (c), CD3 (d) and granzyme B (e).

and variants. Second, *ERBB4*- truncated transcripts have been detected in about one-quarter of cases, which show oncogenic potential. Third, the search for *DUSP22* and *TP63* rearrangements has allowed the identification of three subcategories amongst ALCLs ALK$^-$. The cases with *TP63* rearrangement (8%) are associated with an aggressive clinical course and poor response to therapy. The ones with *DUSP22* rearrangement (30%) are characterised by a quite monotonous centroblastic-like morphology, strong positivity for IRF4, and negativity for EMA and cytotoxic markers. They have a very good response to therapy with OS rates comparable to ALCL ALK$^+$, a fact that might be due to an active immunological response of the host against the tumour. Finally, the cases triple-negative (i.e. lacking *ALK*, *DUSP22* and *TP63* rearrangements) have an intermediate prognosis.

Breast implant-associated anaplastic large-cell lymphoma

Breast implant-associated anaplastic large cell lymphoma (BIA-ALCL) occurs in individuals, who usually received a textured breast prosthesis because of reconstructive or cosmetic reasons. The prevalence is low (2.03 per 1 million persons-years). The predisposing conditions have not been established yet. BIA-ALCL shows the same features as ALCL ALK$^-$. No *DUSP22* rearrangement has so far been recorded. Importantly, the TNM criteria have been applied to the staging of BIA-ALCL. The cases confined within the periprostatic capsule and/or infiltrating the mammary gland are cured by the removal *en block* of the prosthesis and peri-prosthetic capsule. Those infiltrating the thoracic wall or with systemic diffusion of the disease have a worse prognosis and may require systemic therapy.

Hodgkin lymphoma

HL accounts for about 10% of lymphoid tumours. Irrespective of the histotype, HL is characterised by the following features: (i) usual onset in the lymph nodes, preferentially cervical ones, (ii) predilection for young adults and (iii) small number of mononucleated and multinucleated tumour cells (Hodgkin and Reed–Sternberg cells) within an exuberant inflammatory milieu and often ringed by rosettes of T lymphocytes (see also Chapter 25). Biological and clinical studies have shown that HL comprises two disease entities: nodular lymphocyte-predominant HL (NLPHL) and classical HL. The ICC has proposed to substitute the term Hodgkin by B-cell (nodular lymphocyte predominant B-cell lymphoma) to emphasise the different cells of origin and nature compared to classical HL. The ICC also recognises the close relationship of this entity with TCRLBCL.

Nodular lymphocyte-predominant B- cell lymphoma/ Nodular lymphocyte-predominant Hodgkin lymphoma

This entity is well characterised with a monoclonal proliferation of germinal centre-derived large multilobulated B-cell cells known as 'popcorn' or LP cells (Figure 20.27). These cells reside in a follicular background with large FDC networks filled with non-neoplastic lymphocytes and histiocytes. One-third of the cases diagnosed in the past as NLPHL were lymphocyte-rich classical HL. These cases represent approximately 5% of HL cases. Patients are predominantly males in the fourth decade of life. LP cells are regularly positive for CD20, CD79a, EMA, BCL6 and CD45. In contrast to Hodgkin and Reed–Sternberg cells of classical HL, tumour cells are positive for Oct-2, BOB.1 and immunoglobulin light and/or heavy chains but – with a few exceptions – lack CD15, CD30 and EBV infection. The tumour cells are ringed by TFH cells that are positive for CD3, CD4, PD-1 and (to a lesser extent) CD57. Histologically most NLPBL have a nodular pattern with the large tumour cells mixed in a background of B cells. In some cases the background of small B cells is substituted by increasing number of T-cells and the nodular pattern may become a more diffuse pattern. These cases have a worse outcome. Tumour cells show clonal *IGHV* gene rearrangement, with a high load of somatic mutations that may be ongoing. Aberrant somatic hypermutations of *PAX5*, *PIM1*, *RHOH* and *MYC* genes are recorded in 80% of cases. This entity is indolent with frequent relapses that usually remain responsive to therapy. Advanced stages have an unfavourable prognosis. Progression to large B-cell lymphoma is seen in 1–14% of cases.

Classical Hodgkin lymphoma

Classical Hodgkin lymphoma is a monoclonal B-cell neoplasm composed of Hodgkin and Reed–Sternberg cells (HRSCs) within a variable mixture of reactive lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells, fibroblasts and collagen fibres (Figure 20.28). Based on the microenvironmental composition, four histological subtypes are distinguished: lymphocyte-rich, mixed cellularity, lymphocyte-depleted and nodular sclerosis (characterised by HRSC variants, known as 'lacunar cells'). The immunophenotype and genetic features of HRSCs are identical in these histological subtypes, whereas clinical features and EBV association vary. Classical HL accounts for 95% of HLs, with a bimodal age curve in resource-rich countries, showing a peak at 15–35 years and a second peak in later life. Classical HL most often involves cervical lymph nodes and in approximately 60% of patients (usually with the nodular sclerosis subtype) causes a mediastinal mass. HRSCs typically represent 0.1–10% of the cellular infiltrate

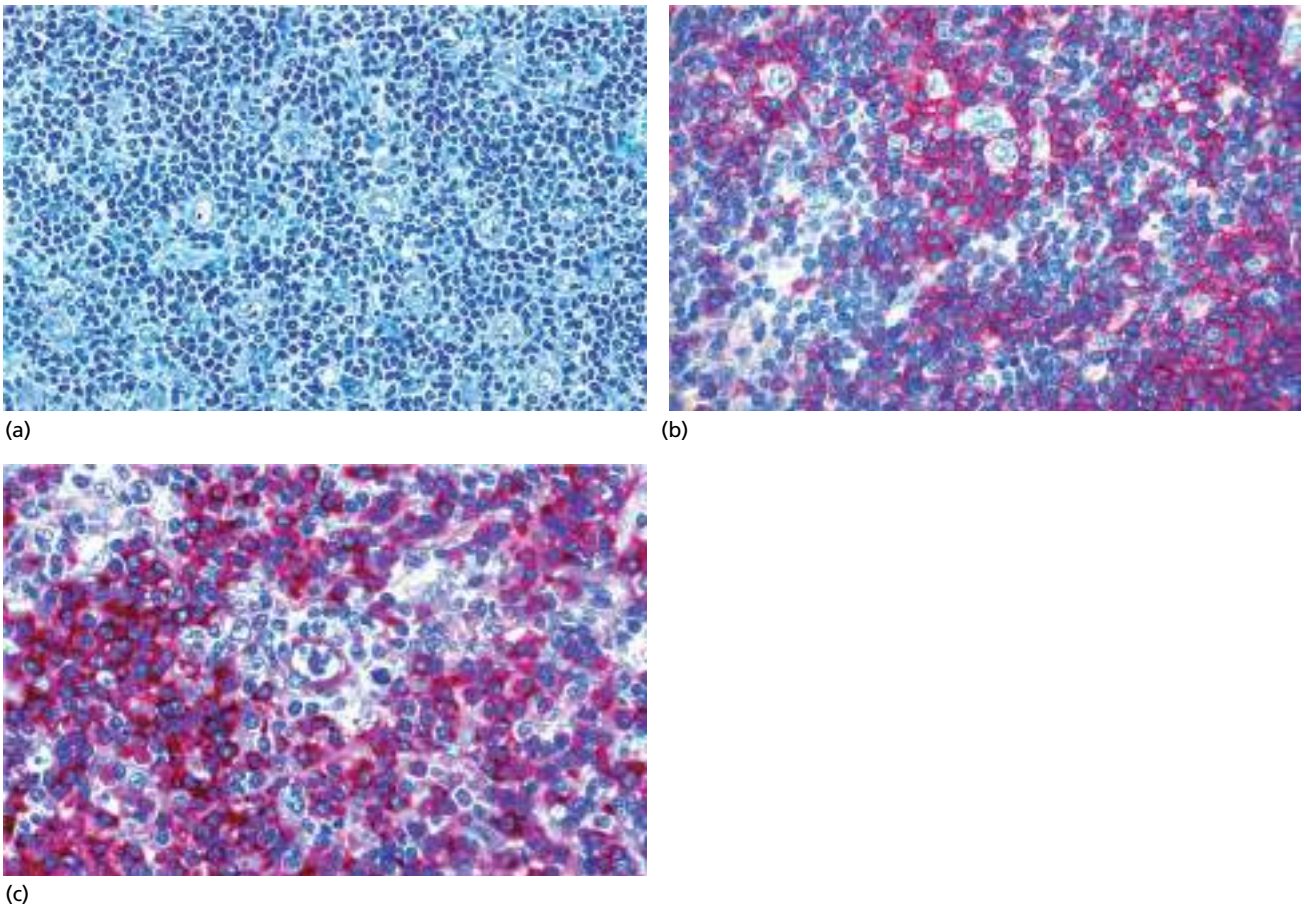

Figure 20.27 Nodular lymphocyte predominant (LP) B-cell lymphoma. (a) Typical LP cells within a milieu of small lymphocytes; (b) The LP tumour cells are surrounded by rosettes of $CD3^+$ T lymphocytes; (c) LP cells and most small lymphocytes express CD79a.

and are CD30 and CD15 positive in nearly all and most cases, respectively. The constitutive expression of CD30 represents the rationale for targeted immunotherapy, extensively applied in the treatment of patients with relapsing/resistant disease. In 30–40% of cases, CD20 may be detected, usually of varied intensity and in a minority of HRSCs. The B-cell nature of neoplastic cells is sustained in about 95% of cases by their expression of PAX5, though weaker than that of reactive B cells. EBV-positive HRSCs show a latency II pattern ($LMP1^+$, $EBNA\text{-}2^-$). Characteristically, the transcription factors Oct-2 and BOB.1 are absent in about 90% of cases, while PU.1 is consistently absent. HRSCs contain clonal IGHV gene rearrangements in more than 98% of cases. The significance of is debatable. Clonal *TCR* gene rearrangements in rare instances. The rearranged *IGHV* genes harbour a high load of somatic hypermutations, usually not ongoing. By analysing microdissected HRSCs, GEP has provided the molecular demonstration of their loss of the B-cell identity, while NGS has shown frequent mutations affecting *STAT6*, *GNA13*, *XPO1* and *ITPKB*. Overall, most cases reveal dysregulation of the JAK-STAT pathway by genetic alterations in multiple genes (also including *STAT3*, *STAT5B*, *JAK1*, *JAK2* and *PTPN1*). The determination of microenvironmental components expressing CD68, CD163 and FoxP3 may assist in better defining the prognosis of patients with classical HL. During the last few years, special attention has been deserved to the expression of

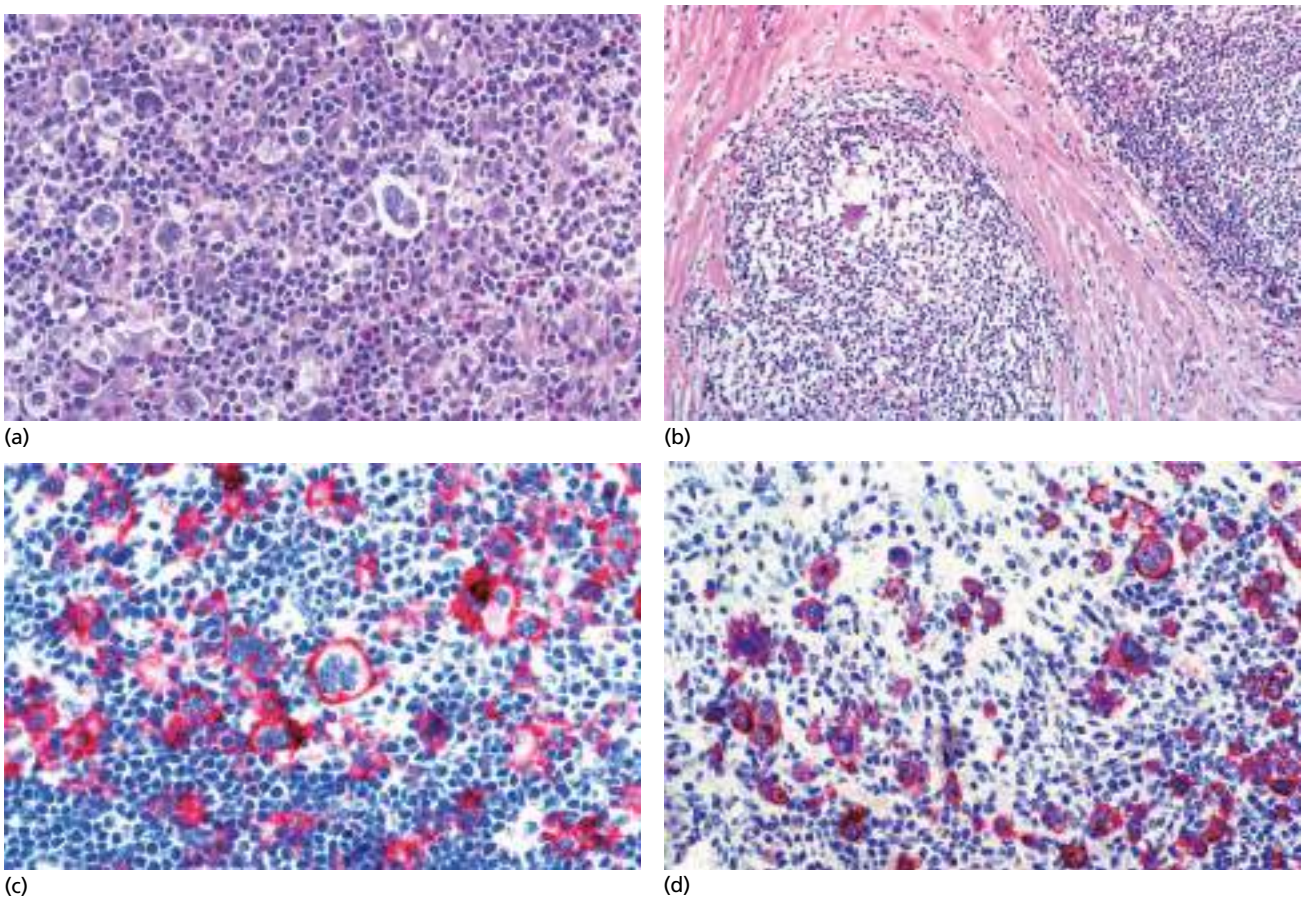

Figure 20.28 Classical Hodgkin lymphoma. (a) Mixed cellularity type; (b) Nodular sclerosing type (H&E, ×100); (c, d) Neoplastic cells positive for CD30 (c) and CD15 (d).

PD-L1 by neoplastic cells (due to copy number alterations of 9p24.1) and tissue macrophages, which contribute to the tumour immune escape along with PD-1$^+$ T-lymphocytes. This has led to the therapeutic usage of immune checkpoint inhibitors in relapsing/resistant cases. More recently, the combination of immune checkpoint inhibitors and anti-CD30 immunotherapy has produced impressive results in patients with relapsing/resistant disease.

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CHAPTER 21

Adult acute lymphoblastic leukaemia

21

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Introduction

Acute lymphoblastic leukaemia (ALL) is an aggressive malignancy of T or B lymphocytes. The onset of ALL is usually rapid, with patients presenting with signs and symptoms of bone marrow failure. Occasionally patients will present with primary involvement of nodal or extranodal sites, with minimal evidence of bone marrow involvement. In these cases the diagnosis is one of lymphoblastic lymphoma (LL). The distinction between ALL and LL is arbitrary. If a patient presents with a mass lesion and less than 25% blasts in the marrow then the diagnosis is one of LL. Where marrow blasts total more than 25% the diagnosis is of leukaemia.

ALL is primarily a disease of childhood, with 75% of cases occurring in children under the age of 10 years at diagnosis. However, although ALL is rare in adults, there is an increasing incidence with age after the age of 40 years. The aetiology of ALL is currently unknown.

A new presentation of ALL is a clinical emergency and should be diagnosed and treated without delay. Most patients present with a combination of symptoms resulting from bone marrow failure, including fatigue and weakness due to anaemia, haemorrhagic complications of thrombocytopenia and/or signs and symptoms of recurrent infection due to neutropenia. Approximately 10% of patients will have obvious signs of organ involvement at diagnosis with splenomegaly, liver impairment, lymphadenopathy, testicular swelling or meningeal syndrome with neurological symptoms. Patients with T-cell disease may have large mediastinal masses and can present with features of superior vena cava obstruction.

Diagnosis of adult ALL

Morphology

In adults, the blood count will usually be very abnormal, with features of bone marrow infiltration. There is frequently a normochromic normocytic anaemia. The white cell count may be increased (59% of cases), normal (14%) or decreased (27%), and there is usually a thrombocytopenia, with one-third of patients having a platelet count of less than 25×10^9/L at diagnosis. Examination of a blood film reveals circulating lymphoblasts in over 90% of cases. Approximately 15% of patients will have a very elevated white cell count above 100×10^9/L and very occasionally the white cell count can be over 500×10^9/L. It is more common to see very high white cell counts in T-cell disease rather than B-cell disease. Most adults will be anaemic at diagnosis, although severe anaemia <80 g/L is unusual.

A bone marrow aspiration is mandatory for the diagnosis of ALL. The bone marrow will be hypercellular with a marked infiltration of leukaemic blasts, which amount to over 25% of total nucleated cells. The majority of cases will have more than 50% infiltration by leukaemic blasts. In some cases, the marrow may be difficult to aspirate due to increased reticulin and a trephine biopsy should be performed. Blastic transformation of chronic myeloid leukaemia should be ruled out by morphologic examination where possible (presence of basophilia, prominent granulocyte component and dwarf megakaryocytes in the bone marrow). However, the distinction between de novo Ph+ ALL and CML in lymphoid blast phase by morphology can be very challenging. Molecular studies may be helpful, i.e. the presence

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

of the p190 *BCR::ABL1* transcript strongly suggests de novo ALL, although the finding of the p210 *BCR::ABL1* transcript is less helpful as it can occur in both scenarios.

Lymphoblasts in both B-cell and T-cell ALL vary from small blasts with scant cytoplasm, condensed nuclear chromatin and indistinct nucleoli to larger cells with moderate amounts of light blue to blue-grey cytoplasm, occasionally vacuolated, dispersed nuclear chromatin and multiple variably prominent nucleoli. Historically, the FAB classification was used to classify ALL blasts based on morphological appearances and size of blasts. This classification was of no relevance prognostically and included mature B-cell disease (Burkitt lymphoma), which has now been removed from classifications of ALL. Burkitt lymphoma in leukaemic phase can clearly be defined by its different immunophenotype (see below) and is now treated on different protocols to ALL.

The current World Health Organization (WHO) classification, shown in Table 21.1, defines ALL and acute lymphoblastic lymphoma together, grouped by their cell of origin, i.e. B cell or T cell. B-cell disease is then further subclassified, based on the presence of recurrent cytogenetic abnormalities that are known to have prognostic value clinically (Table 21.1).

Table 21.1 WHO classification of ALL.

B-lymphoblastic leukaemia/lymphoma
- B-lymphoblastic leukaemia/lymphoma NOS (not otherwise specified)
- B-lymphoblastic leukaemia/lymphoma with high hyperdiploidy
- B-lymphoblastic leukaemia/lymphoma with hypodiploidy
- B-lymphoblastic leukaemia/lymphoma with iAMP21
- B- lymphoblastic leukaemia/lymphoma with *BCR::ABL1* fusion
- B-lymphoblastic leukaemia/lymphoma with *BCR::ABL1*-like features
- B-lymphoblastic leukaemia/lymphoma with *KMT2A* rearrangement
- B-lymphoblastic leukaemia/lymphoma with *ETV6::RUNX1* fusion
- B-lymphoblastic leukaemia/lymphoma with *ETV6::RUNX1*-like features
- B-lymphoblastic leukaemia/lymphoma with *TCF3::PBX1* fusion
- B-lymphoblastic leukaemia/lymphoma with *IGH::IL3* fusion
- B-lymphoblastic leukaemia/lymphoma with *TCF3::HLF* fusion
- B-lymphoblastic leukaemia/lymphoma with other defined genetic abnormalities

T-lymphoblastic leukaemia/lymphoma
T-lymphoblastic leukaemia/lymphoma NOS
Early T-precursor lymphoblastic leukaemia/lymphoma

Cell-surface marker analysis

In diagnosing ALL, the bone marrow – or peripheral blood if the count is high – should be examined using a panel of monoclonal antibodies to T-cell-associated and B-cell-associated antigens, which will identify most cases of ALL. Aberrant expression of myeloid antigens is not uncommon and should not detract from the correct diagnosis. Both B-cell and T-cell ALL are divided into subtypes based on the pattern of cell-surface markers present on the leukaemic blast cells. These subtypes are defined according to their stage of differentiation, although aberrant or asynchronous antigen expression is common. The European Group for the Immunological characterisation of Acute Leukaemia (EGIL) has defined a unified classification for ALL based only on phenotypes. The World Health Organization (WHO) also classifies ALL according to genetic entity – see Cytogenetics below.

B-lineage ALL

The vast majority of cases of B-ALL express HLA-DR, terminal deoxynucleotidyltransferase (TdT) and CD19. CD79a, CD22 and CD34 are frequently – but not always – expressed and levels can vary. B-ALL accounts for approximately 75% of adult cases of ALL and is subdivided into the following groups:

- *Pro-B-ALL* represents approximately 10% of adult ALL; CD10 is negative, as is cytoplasmic immunoglobulin.
- *Common ALL* (c-ALL) is the major immunological subtype in adult ALL, comprising more than 50% of all cases; c-ALL is characterised by the presence of CD10 and absence of cytoplasmic immunoglobulin.
- *Pre-B-ALL* is characterised by the expression of cytoplasmic immunoglobulin, which is absent in c-ALL, but is identical to c-ALL with respect to the expression of all other cell markers.
- *Mature B-cell ALL* is found in approximately 4% of adult ALL patients and is now classified as Burkitt lymphoma rather than ALL. The blast cells are negative for TdT and express surface antigens of mature B cells, including surface membrane immunoglobulin.

T-lineage ALL

T-cell ALL accounts for approximately 25% of adult ALL. Cells are TdT+, in addition to cytoplasmic CD3+ and CD34+. All cases express the T-cell antigen gp40 (CD7) and they may, according to their degree of T-cell differentiation, express other T-cell antigens, for example the E rosette receptor (CD2) or the cortical thymocyte antigen T6 (CD1). A minority of T-cell ALL blast cells express CD10 together with T-cell antigens. In most cases of T-cell ALL, one or more of the T-cell receptor (*TCR*) genes are rearranged. These properties make it possible to

classify T-cell ALL according to their stage of intrathymic differentiation:

- *Early T-precursor ALL* accounts for 6% of adult ALL and is associated with inferior outcomes compared to other types of T-cell ALL; it shows characteristic T-cell markers (cyCD3 and CD7), but no further differentiation markers.
- *Thymic (cortical) T-ALL* is the most frequent subtype of TALL (10% of ALL). It is characterised by the expression of CD1a; surface CD3 may be present. Since this subtype is associated with a better prognosis, its identification is of particular importance.
- *Mature T-ALL* has a frequency of 6%; the blast cells do not express CD1a, but they are positive for surface CD3.

Genetics

Techniques for diagnosis

ALL is characterised by somatic genetic alterations which initiate and drive leukaemogenesis and have prognostic relevance. These lesions consist of recurring chromosome abnormalities, kinase-activating gene fusions, or so-called 'Ph-like' ALL. Cytogenetic and/or other relevant genetic screening to identify these lesions is mandatory at diagnosis. Whilst examination of metaphases is now less commonly carried out, fluorescence *in situ* hybridisation (FISH) with specific probes e.g. for *BCR::ABL1*, *MLL::AF4, ETV6::RUNX1* is a common, rapid and effective way to screen for known translocations. Screening by reverse transcriptase polymerase chain reaction (RT-PCR) for the potential *BCR::ABL1* transcripts, p190 and p210, should also be performed at diagnosis. Using standard techniques, about 20–30% of B-ALL cases cannot be classified into current WHO genetic subgroups shown in the table. Whole genome sequencing, RNA sequencing and other genomic studies such as SNP (single nucleotide polymorphism) arrays and array CGH (comparative genomic hybridisation) have revealed several recurring genetic abnormalities amongst those without the 'typical' driver lesions. Examples include fusions involving *MEF2D*, *DUX-4* or *ZNF384*.

Summary of most common genetic lesions

Up to 70% of children between 1 and 10 years presenting with ALL will have so-called 'good-risk' cytogenetics with the presence of either hyperdiploidy (between 51 and 65 chromosomes) or the translocation t(12;21)(p12;q22), resulting in the fusion product *ETV6::RUNX1* (see Chapter 22). By contrast, 'good-risk' cytogenetic abnormalities are rare in adult ALL and decrease with increasing age. However, when good-risk aberrations are detected at diagnosis in adults they carry the same prognostic value as they do in children. Conversely, the incidence of poor-risk cytogenetics, such as hypodiploidy and *BCR::ABL1* (Philadelphia chromosome)-positive ALL generally rises with age, although there appears to be a plateau in incidence above the age of 50 years.

The poor prognostic impact of the finding of a t(9;22) leading to the *BCR::ABL1* fusion – approximately 25% of adults ALL – has long been recognised. The so-called Philadelphia chromosome t(9;22)(q34;q11), results from a translocation involving the *BCR* gene on chromosome 22 and the *ABL1* gene on chromosome 9. One-third of adult patients with *BCR::ABL1+ ALL* will have the 'Major'-*BCR* rearrangement (resulting in a 210-kDa protein), as seen in chronic myeloid leukaemia, detectable by PCR. The remaining two-thirds will have the 'minor'-*BCR* rearrangement (resulting in a 190-kDa protein). Patients with *BCR::ABL1+* ALL were – in the past – less likely to enter complete remission (CR) with CR rates of 70–80%. Such patients had an overall survival of 15–20%.

ALL treatment protocols have typically assigned patients with *BCR::ABL1+* to 'very high-risk' protocols and a myeloablative transplant has typically been offered, even when sibling donors have not been available. The advent of tyrosine kinase inhibitors has completely changed the outcome for this subset of patients, as discussed below.

The translocation t(4;11)(q21;q23), involving the *KMT2A* gene on chromosome 11q23, has also been recognised as carrying a poor prognosis in adult ALL. In an analysis of data from 1522 adult patients participating in the UKALLXII/ECOG2993 study, the following four groups of patients with distinct karyotypes were shown to have markedly inferior rates of event free survival and overall survival when compared to the whole cohort: t(9;22), t(4;11), complex karyotype (defined as five or more chromosomal abnormalities) and low hypodiploidy/near triploidy. Amongst patients with *BCR::ABL1*-negative ALL, the prognostic relevance of these abnormalities was independent of other previously known poor prognostic factors, such as age and presenting white cell count. This was the first demonstration that cytogenetic subgroups other than *BCR::ABL1* can be used for risk stratification of adults with ALL. It is highly likely that additional risk stratifications based on genetic abnormalities at diagnosis will be part of routine clinical practice within the next 5–10 years.

Minimal residual disease

Morphological detection of remission post treatment has very limited sensitivity of 1–5% and a patient in a morphological complete remission may still bear a considerable disease burden. It is now possible to quantitate treatment response very accurately and reproducibly, to the level of 1 leukaemic cell in 10,000 using various methods, as shown in Table 21.2. The most standardised method identifies patient-specific immunoglobulin heavy chain and *T-cell* receptor gene rearrangements, which can then be quantified by real-time polymerase chain reaction (PCR) in a patient-specific assay with a sensitivity of 10^{-5} (1 in 100,000 cells). Flow cytometry can also be used to determine a

Table 21.2 Comparison of MRD assays in ALL.

Method	Applicability	Sensitivity of leukaemia cell detection	Specimens needed
Flow cytometry	Approx. 95%	1 in 10^4	Fresh cells
RQ-PCR for Ig/*TCR* rearrangements	Approx. 90%	1 in 10^5	DNA
RQ-PCR for fusion gene transcripts such as *BCR::ABL1*	Depends on frequency of abnormality; *BCR::ABL1* is 20–25%	1 in 10^5	RNA
High throughput sequencing to quantify Ig/*TCR* rearrangements	Approx. 90%	1 in $10^{5–6}$	DNA

disease-associated immunophenotype at diagnosis, which can also form the basis of an minimal residual disease (MRD) assay. A diagnostic sample is required in both cases. Where there is a known molecular abnormality such as *BCR::ABL1*, this can also be quantified, using a housekeeping gene as a reference standard for PCR, to determine the level of residual ALL with similar sensitivity. Both molecular and flow cytometry techniques for quantification of MRD are labour intensive when performed properly and should be carried out in reference laboratories, according to standardised protocols. Studies in both childhood and adult ALL have shown a significant correlation between MRD levels and subsequent relapse risk (see Chapter 22). However, it should be noted that interpretation of MRD results is always protocol-dependent. It is not yet known whether high-risk interventions such as stem cell transplant can overcome the poor prognostic significance of residual MRD, and this question is the current focus of several ongoing large Phase III clinical trials.

It is also possible to use MRD analysis to monitor the progress of patients in remission in order to detect impending relapse – the time window between molecular and haematological relapse is on the order of a few months, possibly yielding an opportunity for early intervention. However, the clinical potential of treatment prior to haematological relapse is not yet clear. Table 21.2 shows a comparison of different methods for detection of MRD with advantages and disadvantages.

Prognostic and predictive factors in ALL

A prognostic factor is a clinical or genetic characteristic that is objectively measurable and that provides information on the likely outcome of the ALL in an untreated person. A predictive factor is a clinical or genetic characteristic that provides information on the likely benefit from treatment. Prognostic and predictive factors are summarised in Table 21.3. A number of clinical and genetic factors have been clearly identified as poor prognostic factors in ALL; advancing age, higher presenting white cell count at diagnosis, presence of CNS disease, certain cytogenetic abnormalities, specific immunophenotypes and specific genetic abnormalities such as *IKZF1* (Ikaros gene) deletions. Response to initial therapy, early steroid response and quantification of minimal residual disease at protocol-defined time points are validated predictive factors.

Most current treatment strategies and trials take some or all of these factors into account in defining a risk status in ALL. There is no universally accepted algorithm by which risk status is measured in all cases. Additionally, whilst there is sometimes a relationship between these poor risk factors (for example, patients with T-ALL often have very high presenting white cell counts and CNS disease), the poor outcome portended by initial presence of poor prognostic factors can be superseded by a good treatment response, as documented by having a negative MRD status after induction. Hence, how we vary our therapeutic strategy in response to prognostic and predictive factors will change as studies identify new markers and define their inter-relationships. Figure 21.1 (kindly provided by Anthony Moorman and Christine Harrison, Leukaemia Research Cytogenetics Group, Northern Institute for Cancer Research, Newcastle University, UK) is based on an analysis of adults entered into UK trials of ALL. It shows the genetic heterogeneity of adult ALL, detailing some of the relationships between risk groups and further, their relationships with outcome. Table 21.3 highlights some of the factors that are currently taken into account internationally when making decisions about prognosis and approach to treatment.

Initial approach to a patient with ALL

Clinical presentation

The majority of adult patients with ALL will present with clinical symptoms of bone marrow failure. The history is often very short, i.e. a few weeks and patients frequently report a very rapid progression of their symptoms. The commonest symptoms

Table 21.3 Factors commonly used to predict outcome or stratify therapy.

Factor	Poor risk	Neutral or good risk
Presenting WCC	$>30 \times 10^9$/L B ALL $>100 \times 10^9$/L T-ALL	Low presenting WCC
Age	Worse outcome with advancing age	Younger age
Immunophenotype	T-cell phenotype better outcome than B-cell phenotype Early T precursor ALL (ETP) Expression of CD20 (some studies)	Expression of myeloid antigens
Cytogenetics	t(9;22) (*BCR::ABL1*) t(4;11) (*MLL::AF4*) Complex karyotype (five or more chromosomal abnormalities) Low hypodiploidy/near triploidy	t(12;21) (*ETV6::RUNX1*) Hyperdiploidy
Specific genetic abnormalities	*IKZF1* deletions, particularly in Ph+ ALL	*NOTCH1* and *FBXW7* mutations in T-ALL
Early response to steroid	Poor initial response to steroid therapy	
MRD	Positive signal at protocol-relevant time point predicts less good outcome	Negative signal at protocol-relevant time point

that cause the patient to seek medical advice are recurrent infection or petechiae or other haemorrhagic problems. Weakness and fatigue due to anaemia are also common presenting features. Approximately half of all patients will have findings of lymphadenopathy, splenomegaly and/or hepatomegaly at presentation. According to data from two large German trials, 14% of patients will have a mediastinal mass at presentation and the majority of these (85%) will subsequently be shown to have T-cell disease. Examination of the cerebral spinal fluid (CSF) at diagnosis reveals leukaemic blasts in 7% of patients, although only 4% have central nervous system (CNS) symptoms such as headache, vomiting, lethargy and cranial or peripheral nerve dysfunction.

Almost any organ can be infiltrated by leukaemic blasts at diagnosis and approximately 10% of patients will have demonstrable involvement clinically. Pleural effusions are most common and tend to occur in association with mediastinal masses in T-cell disease. Bone pain at presentation is rare in adults compared to children, with bone lesions being demonstrated in 1% of adult patients. Involvement of the testes is very rare (<1%) in adult male patients. Leukaemic infiltration of other organs such as lung, kidney and skin is rarely observed and tends to be associated with poorer outcomes.

Diagnostic approach

A new diagnosis of ALL is a medical emergency and should be investigated and treated without delay. All patients require a detailed history to be taken alongside a thorough medical examination. The admitting clinician needs to pay particular attention to signs and symptoms of pre-existing infection, including fungal infection. Patients should be asked specifically about the presence of symptoms of sinusitis and haemoptysis. A careful neurological examination is also mandatory.

Investigations at diagnosis must include a full blood count, blood film examination and bone marrow. Urgent biochemical testing, including analysis of urate levels, calcium, renal function, liver function and lactate dehydrogenase (LDH) are required. A coagulation screen should also be performed.

Many modern protocols include the use of monoclonal antibodies such as rituximab in induction. Testing of hepatitis B and C serology prior to the start of therapy is mandatory if rituximab is to be used. It is also good practice to check the HIV status of a patient before starting induction chemotherapy.

A lumbar puncture should be performed in patients at diagnosis, if there is clinical evidence of CNS involvement with leukaemia, to assess whether or not there is leukaemic infiltration of the CSF. Ideally the CSF should be examined by flow cytometry. If there is no clinical suspicion of CNS involvement then many protocols start systemic chemotherapy to lower the disease burden before the first lumbar puncture is performed to reduce the risk of introducing disease into the CSF. Intrathecal methotrexate should always be given at the first lumbar puncture.

A chest X-ray (CXR) is mandatory for all newly diagnosed patients with ALL, but computerised tomography (CT) scans are not routinely required. However, where there is clinical evidence of lymphadenopathy, mediastinal widening or organ infiltration, a CT scan should be performed.

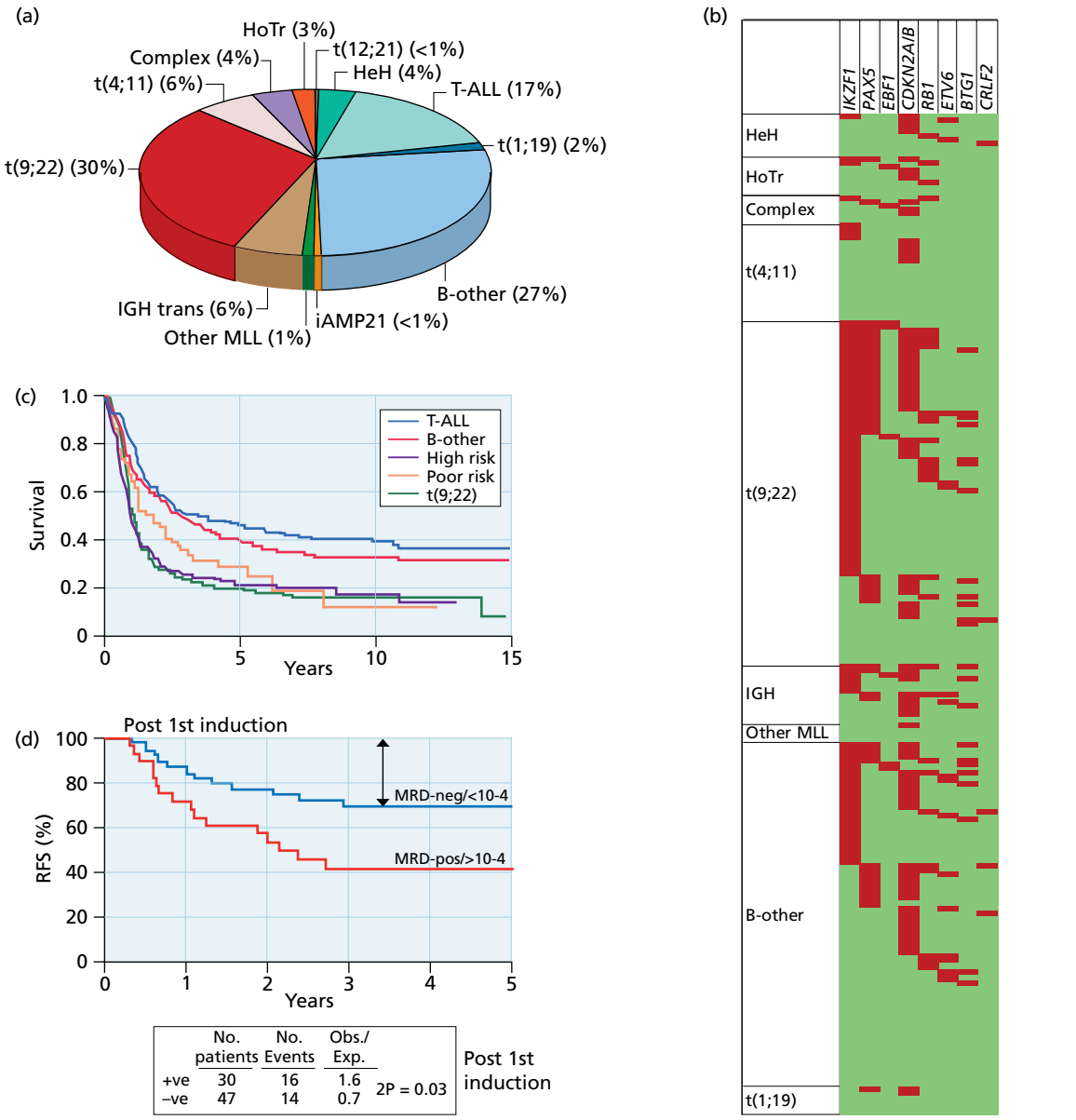

Figure 21.1 *Genetic and outcome heterogeneity within adult ALL.* (a) Distribution of 1014 UKALLXII/UKALL14 patients (25–60 years) according to primary genetic subtype. (b) Correlation of eight key secondary copy number alterations with the main primary genetic subtype in 257 adults (25–60 years). (c) Outcome of 1065 UKALLXII/ECOG2993 adults (25–60 years) by genetic subgroup. The high-risk group comprises those abnormalities which are now used to direct high-risk treatment on UKALL14: t(4;11), HoTr, complex. The poor-risk group comprises those aberrations which have recently been identified as being associated with a poor outcome, but are not currently used to direct treatment: *IKZF1* deletions, *CRLF2* rearrangements and *IGH* translocations. The B-other group comprises all cases with successful cytogenetics but that do not harbour an established abnormality. NB: Patients with rarer subtypes e.g. t(12;21), HeH, t(1;19) have been excluded for clarity. (d) Disease-free survival of t(9;22)-negative B-cell precursor UKALLXII patients according to Ig/TCR-based MRD levels post first induction. HeH, High hyperdiploidy (51–65 chromosomes), t(12;21), t(12;21)(p13;q21)/*ETV6::RUNX1*; HoTr, Low hypodiploidy/near-triploidy (30–39/60–78 chromosomes; complex karyotype (five or more unrelated aberrations in the absence of another primary abnormality), t(4;11), t(4;11)(q21;q23)/*MLL::AF4*; t(9;22), t(9;22)(q34;q11)/*BCR::ABL1*; *IGH* trans, translocations involving the *IGH*/14q32 locus; Other *MLL*, translocations involving *MLL* except t(4;11); iAMP21, intrachromosomal amplification of chromosome 21, t(1;19), t(1;19)(q23;p13)/*TCF3::PBX1*. (Source: Anthony Moorman and Christine Harrison, Leukaemia Research Cytogenetics Group, Northern Institute for Cancer Research, Newcastle University, UK. Reproduced with permission.)

Supportive care

Before starting treatment, patients should be well hydrated and receive appropriate medication to prevent urate nephropathy (see also Chapter 55). ALL can be exquisitely sensitive to small amounts of treatment, even in the steroid prephase, and tumour lysis syndrome is not uncommon. Allopurinol should be started 24 hours before induction therapy with steroids or chemotherapy and should be continued for a minimum of 5 days. Rasburicase should be considered as an alternative to allopurinol if the white cell count is high ($>100 \times 10^9$/L), if there is bulky nodal or extranodal disease or if the patient already has impaired renal function. It should be noted that rasburicase can cause haemolytic anaemia in patients with glucose-6-phosphate dehydrogenase deficiency (G6PD). Consideration should be given to G6PD testing in patients prior to commencing rasburicase.

Due to the combination of myelosuppressive chemotherapy drugs and steroids that patients with ALL receive, they are at high risk of infectious complications from treatment, particularly during the induction phase of therapy. Adherence to strict anti-infective strategies is important. All patients should receive prophylaxis against herpes simplex virus and varicella zoster reactivation with aciclovir or an equivalent treatment. Patients also require prophylaxis against *Pneumocystis jirovecii* from the start of therapy. The recommended prophylaxis is cotrimoxazole. It should be noted that allergy to cotrimoxazole is not uncommon and alternative agents such as pentamidine or dapsone should be used for these patients.

Antifungal prophylaxis is strongly recommended for all patients on ALL therapy from induction until the start of maintenance. Azoles should be avoided when a patient is concurrently receiving vincristine due to the high risk of potentiation of the toxicity of vincristine, particularly neurotoxicity. There is no clear evidence currently about which antifungal prophylaxis regimen should be followed and local policies should apply.

The use of granulocyte-colony stimulating factor (G-CSF), given in parallel with induction chemotherapy, has been shown to reduce the incidence and severity of infections during the first 4 weeks of therapy. Its use has been associated with reduced induction mortality due to infection in several studies. Consequently, the addition of G-CSF in induction (and all subsequent cycles of therapy except maintenance therapy) is also strongly recommended for all adult patients on ALL therapy.

Patients with newly diagnosed ALL have a high risk of venous thromboembolism (VTE), particularly during induction therapy, even if they do not receive an L-asparaginase-containing regimen. This prothrombotic state can exist despite a coexisting bleeding tendency due to thrombocytopenia. It is strongly recommended that all hospitalised patients receive low molecular weight heparin (LMWH) as prophylaxis against VTE unless there are contraindications e.g. a platelet count below 50×10^9/L or clinical evidence of active bleeding.

A checklist of procedures required prior to commencing anti leukaemia therapy in a patient with newly diagnosed ALL is given in Table 21.4. General aspects of supportive care in haematology are also discussed in Chapter 55.

Specific treatment of ALL

The standard treatment for adult ALL has so far not been defined. It is strongly recommended that, where available, all patients with newly diagnosed ALL are offered treatment within the context of a large Phase III clinical trial. Many countries have well-organised study groups, which focus on developing, running and analysing data from clinical trials. Increasingly, there is stronger, regular communication with practical collaborations between clinicians and scientists to use patient specimens coupled to clinical outcome data to ask questions about the genetics of ALL, using, for example, next-generation sequencing approaches. Treatments have been developed on regimens that have evolved over time, with the gradual introduction of novel agents and new approaches, such that current interventions are now based on a strong literature with clinical and scientific contributions from numerous large international clinical trials.

The aim of initial treatment is to achieve complete remission (CR), which is currently defined on a morphological basis of less than 5% blasts in the bone marrow in the presence of overall haematological recovery (neutrophils $>1.0 \times 10^9$/L, platelets $>100 \times 10^9$/L).

Corticosteroids are amongst the most important drugs in the initial treatment of ALL. Paediatric trials have shown improved outcomes when dexamethasone is used as opposed to prednisolone. Dexamethasone does have better anti leukaemic activity than prednisolone *in vitro*; it has better penetration of the CNS and causes fewer thromboembolic events. Although there are less data in adult ALL showing a survival advantage with dexamethasone compared to prednisolone, many adult regimens now have dexamethasone as the preferred corticosteroid of choice in induction. A generalised schema for treatment is shown in Figure 21.2.

Prephase

A steroid prephase for 5–7 days prior to commencing chemotherapy can be very beneficial in the treatment of ALL. Patients will often have a good partial response to steroids. Early steroids can reduce disease burden and improve performance status allowing the patient to be in a better position to receive induction chemotherapy. Steroids are also very useful when there is liver infiltration with ALL and vincristine and anthracyclines cannot be administered as planned due to high levels of serum bilirubin. There are rare situations where a steroid prephase is not recommended, due to the need for emergency anthracycline-based chemotherapy, such as superior vena cava obstruction due to a large mediastinal mass. The risk of tumour lysis is always high in these situations and rasburicase should be given prior to chemotherapy.

Table 21.4 Suggested checklist for the initial assessment of an adult patient with ALL.

Procedure	Mandatory (M)/ Recommended (R)
Clinical assessment:	
• Full history	M
• Clinical examination including examination of the CNS	M
• Height, weight and body surface area	M
• Assessment of performance status	R
Investigations	
• Full blood count and film	M
• Coagulation screen	M
• Biochemical profile, LDH and urate levels	M
• Blood cultures if patient is febrile	M
• Check G-6-PD status if rasburicase to be used	R
• Pregnancy test for women of childbearing age	M
• HIV testing	R
• Hepatitis B and C serology if monoclonal antibody therapy, e.g. rituximab, is part of the planned therapeutic regimen	M
• Bone marrow aspirate:	M
◦ Morphology	M
◦ Flow cytometry	M
• Cytogenetics (G-banding and FISH analysis)	M
• Molecular diagnostics	M
• Evaluation for MRD marker	M
• Lumbar puncture if evidence of CNS involvement	M
◦ Examination of CSF for blasts by cytospin	M
◦ Flow cytometry of CSF	R
• CXR	M
• CT of neck, thorax, abdomen and pelvis if evidence of nodal/extramedullary disease	R
• CT head if clinical evidence of CNS/ophthalmic disease	M
• Tissue typing of patient and siblings (unless a young adult being treated on a paediatric protocol)	R
Pretreatment	
• Gain IV access. Avoid central line in induction if possible due to increased risk of VTE	R
• Adequate hydration. Use IV fluids if WCC is high or significant extramedullary disease	M
• IV antibiotics if patient is febrile	M
• Prevention of urate nephropathy with allopurinol or rasburicase	M
• Infection prophylaxis against:	
◦ Pneumocystis jrovecii	M
◦ *Varicella* zoster	M
◦ Fungi	R
• Growth factor support during induction	R
• Thromboprophylaxis for in-patients if platelets $>50 \times 10^9$/L	R
Treatment	
• Steroid prephase for 5–7 days	R
• Consider entry into clinical trial if available	R

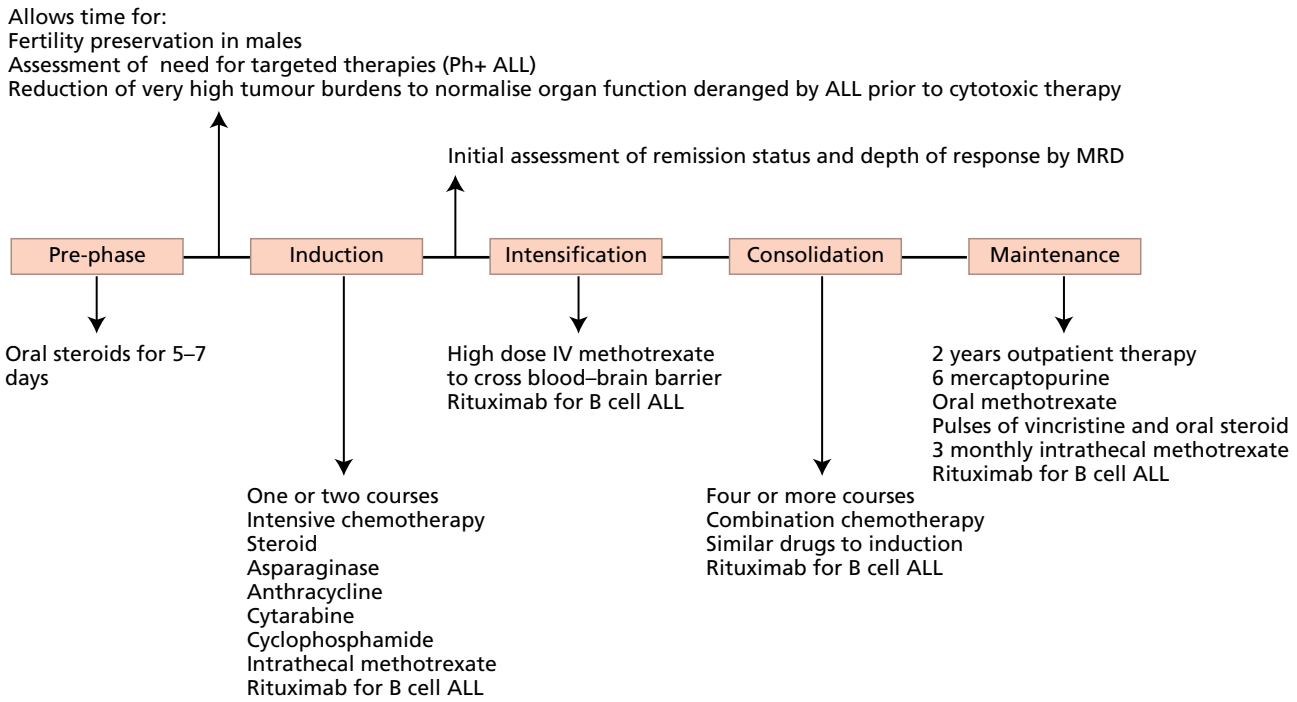

Figure 21.2 A generalised schema for treatment of adult acute lymphoblastic leukaemia.

Induction therapy

The primary goal of induction therapy is complete eradication of ALL cells from the blood, bone marrow, CNS and other extramedullary sites when initially involved. Ideally, CR should be achieved as early as possible with minimal toxicity. Nonetheless, toxicity is often high, especially by contrast to that seen when treating children and many adult patients are hospitalised for weeks to months. There is no universally agreed induction protocol, but most adult treatment regimens are broadly similar in the drug dosing and scheduling. Many protocols use two phases of induction, with the second part being applied regardless of the initial response. Induction regimens in adult ALL are composed of steroid, vincristine, anthracycline (usually daunorubicin or doxorubicin) and L-asparaginase. Cyclophosphamide and cytarabine may also be included. It is also now standard practice to include the anti CD20 monoclonal antibody, rituximab in induction for patients with CD20-positive B-cell ALL, following the demonstration of benefit in a randomised trial conducted by the French GRAALL group. With modern protocols, CR rates following induction therapy are high with over 90% of unselected adults with ALL achieving an initial good response to treatment.

Toxicity during induction

Induction is generally the most risky time during a patient's treatment and they should be monitored closely and seen regularly for clinical assessment. Induction death rates range from 2 to 20%, depending on the protocol used and the patient's age. Most induction deaths are due to severe bacterial sepsis, with fungal infection also posing a significant risk. Steroids often mask signs and symptoms of infection, so astute clinical judgement is required. All patients should have access to a specialist haematology unit 24 hours a day, and they must be treated promptly with intravenous antibiotics for signs of neutropenic sepsis. Any patient with a persisting fever should be investigated for invasive fungal infection.

In addition to the risk of infection, patients are also at risk of problems relating to specific drugs used in induction. L-asparaginase is one of the most effective and important drugs in the treatment of ALL, but it is also one of the most difficult drugs to dose and manage. A major problem with L-asparaginase is the occurrence of coagulation abnormalities during treatment.

L-asparaginase therapy results in reduced levels of fibrinogen and antithrombin levels, but there is little or no evidence that infusions of fresh frozen plasma or fibrinogen can correct the coagulation abnormalities and prevent thrombosis or bleeding. Conversely, there is also some evidence that giving fresh frozen plasma replenishes the asparaginase pool that the drug is being used to deplete, negating its effectiveness. Consequently, the use of plasma-derived products to correct coagulation abnormalities is not recommended unless the patient is actively bleeding.

Despite deranged coagulation being the abnormality measured in the laboratory, an increased risk of VTE is the main clinical problem with L-asparaginase. Data indicate that 10–20% of adults receiving L-asparaginase will develop a VTE. As a

consequence, strategies to reduce the risk of VTE are important. It should be noted that with modern adult ALL protocols, patients might not be thrombocytopenic throughout the whole of induction. Hospitalised patients should be given LMWH thromboprophylaxis if there are no contraindications as discussed above. The presence of a central venous catheter increases the risk of VTE and many centres avoid placing a central venous catheter in induction until the patient has achieved a remission. There are little data available on how to manage post L-asparaginase VTE. When a catheter-related thrombosis occurs then the treatment of choice is LMWH. Treatment of a central venous thrombosis is less clear. Patients should be treated with either LMWH or unfractionated heparin. Adequate monitoring of the heparin dosing using the anti-Xa assay should be performed as patients may be resistant to heparin due to depletion of antithrombin. In this situation, replacement of antithrombin is likely to be valuable, and specialist coagulation advice should be sought.

There are data showing inferior outcomes for patients who develop VTE during induction therapy for ALL despite the fact that the thromboses that occur with L-asparaginase therapy are not usually fatal. It is assumed that the occurrence of a thrombosis results in treatment delays, which are then detrimental to the patient in the longer term.

Patients with previous L-asparaginase-related VTE can be given further L-asparaginase with anticoagulation cover during consolidation therapy.

It is common for patients on L-asparaginase to develop transaminitis and hyperbilirubinaemia during induction therapy. If the patient develops acute sepsis then this will exacerbate the problem. All non-essential drugs that are liver metabolised should be withheld. With supportive care, the liver abnormalities usually fully resolve, but severe treatment delays often result.

Pancreatitis is a rare, but life-threatening, complication of L-asparaginase, which is more common in children than in adults. Serum amylase levels should always be checked in a patient post L-asparaginase therapy who is complaining of abdominal pain. The development of pancreatitis is a contraindication to further use of the drug.

Consolidation therapy

Consolidation therapy is given to patients in CR with the aim of eliminating residual leukaemia cells and to prevent the re-emergence of drug-resistant disease. Consolidation therapy has typically consisted of several cycles of treatment with multiagent chemotherapy, including CNS-directed therapy and further doses of L-asparaginase. Modern adult protocols frequently contain at least one delayed intensification block of therapy, which constitutes a modified re-induction schedule mirroring phases 1 and 2 of induction therapy. Rituximab should also be incorporated into consolidation for patients with CD20-positive B-cell ALL. Smooth progression between courses of therapy to minimise delays is likely to be important, although there are usually specific requirements for peripheral blood count recovery between courses. Slow recovery between courses should be taken seriously as it may indicate actual or impending relapse. Consolidation is generally better tolerated than induction, partly because the patient will have achieved CR and will begin therapy with adequate bone marrow function. Many patients can receive at least some elements of their consolidation therapy as outpatients.

With the approval of the CD19-CD3-directed bispecific antibody, blinatumomab, for use in patients with MRD persisting after induction at the level of 0.01% or greater, it is possible and desirable to deliver this agent as a non-chemotherapy consolidation to patients with higher levels of residual disease after induction.

Maintenance therapy

Maintenance therapy remains obligatory for all adult patients who have not undergone an allogeneic stem cell transplant as part of their treatment for ALL. Studies evaluating the omission of maintenance therapy have concluded that this results in inferior outcomes. The standard approach to maintenance includes daily 6-mercaptopurine and weekly methotrexate with pulses of vincristine and steroids for a period of approximately 2 years post consolidation. However, individual protocols vary. Rituximab should also be incorporated into maintenance therapy for patients with CD20 positive B-cell ALL, to a total of 14–16 cycles across the entire ALL treatment protocol. The aim of maintenance is to eliminate minimal residual disease and dosing of the chemotherapeutic agents needs to be strictly adjusted to induce moderate myelosuppression, whilst not putting the patient at risk of neutropenic sepsis or haemorrhage. CNS-directed prophylaxis with intrathecal chemotherapy should continue during maintenance. Pneumocystis prophylaxis and herpes zoster prophylaxis should also continue throughout the maintenance period (and for at least 6 weeks post completion of chemotherapy), although antifungal prophylaxis is no longer required.

CNS-directed therapy

Without CNS-directed treatment, approximately one-third of patients with ALL will experience a CNS relapse. Risk factors include T-cell disease, high white cell count at diagnosis, CNS disease at diagnosis, other extramedullary sites of disease and high-risk cytogenetics such as Ph+ ALL. Traditionally intracranial radiotherapy was used to prevent CNS relapse. Whilst effective, this resulted in long-term complications including neurocognitive sequelae, second cancers and endocrinopathies. In adults, several trials have reported CNS recurrence rates of 5–7% when a combination of high-dose methotrexate ($\geq 3\ g/m^2$) and intrathecal chemotherapy is used

without additional cranial radiotherapy. The use of dexamethasone in induction has also been shown to reduce CNS relapse rates in children and young adults. In most adult and paediatric protocols, CNS irradiation is no longer used in the prophylactic setting.

Intravenous (IV) and intrathecal (IT) methotrexate is the mainstay of therapy targeted at preventing CNS relapse in patients with ALL. It is commonly given via the lumbar puncture route throughout all phases of treatment, including maintenance therapy. Cytarabine and steroid can also be given intrathecally and are sometimes given with methotrexate as 'triple therapy', although there are no data suggesting the superiority of adding the additional drugs.

The precise number of lumbar punctures needed during therapy is not defined. In adult ALL, between 10 and 20 doses of IT therapy are given, dependent upon treatment protocol. In addition, many adult protocols include an intensification block of high-dose methotrexate given at doses $\geq 3\,g/m^2$.

It is important that only non-cutting lumbar puncture needles are used to administer IT chemotherapy during ALL therapy. The commonly available cutting needles that are often used for diagnostic procedures are more likely to cause a dural tear and subsequent CSF leak which can result in post lumbar puncture headache. The potentially serious complication of bilateral hygromas developing as a consequence of delivering repeated IT chemotherapy via a cutting needle has also been reported in adult patients receiving ALL therapy. The incidence of both post lumbar puncture headache and development of hygromas can be significantly reduced with the use of non-cutting needles.

One of the most dramatic complications of ALL therapy is methotrexate (MTX)-induced encephalopathy. Although this side effect is rare, it is very frightening for patients and their relatives when it does occur. MTX-induced encephalopathy is more frequently seen in young adults than older adults and usually occurs when IT methotrexate is being given during a phase of intravenous cytarabine. It may present with fits, focal neurological signs or stroke-like syndrome with or without impaired consciousness. It occurs within 1 day to 3 weeks after exposure to IT methotrexate and hence it is not always the most evident explanation for the signs and symptoms. Fortunately, patients usually make a full recovery, although there have been case reports where a degree of neurological damage has persisted. It is important to differentiate MTX-induced encephalopathy from cerebral sinus thrombosis with the appropriate imaging. The presence of ALL within the CNS also needs to be ruled out in any patient presenting with a sudden onset of neurological symptoms. In the case of MTX-induced encephalopathy, IT methotrexate should not be given whilst the patient is receiving systemic cytarabine. It can often safely be reintroduced later in the protocol, but this should be approached on a case-by-case basis. Alternatively, the patient can be given cytarabine intrathecally, in association with IT steroids.

Treatment of CNS disease at diagnosis

For those patients where there is evidence of CNS disease at diagnosis, the current advice is to give twice-weekly IT chemotherapy (methotrexate with or without cytarabine and steroids) alongside induction therapy until the CSF is clear of lymphoblasts on two consecutive samples. Cranial radiotherapy post induction remains the standard treatment of CNS disease for this small group of patients. However, if the patient is due to receive allogeneic stem cell transplant with total body irradiation conditioning, CNS irradiation can be withheld. Further IT methotrexate should be given in consolidation and maintenance post radiotherapy as per protocol

Stem cell transplantation

Myeloablative allogeneic haemopoietic stem cell transplantation (alloHSCT) is undoubtedly one of the most effective anti-ALL therapies available and there is a clear graft-versus-ALL effect. One of the biggest ever studies of adult ALL (UKALL12/E2993) demonstrated a survival advantage for myeloablative sibling alloHSCT over chemotherapy in a 'donor versus no donor', intention-to-treat analysis. Unfortunately, it is also the most toxic approach within our therapeutic scope. It has played a significant role in the treatment of ALL over many years, in some country's approaches, being reserved for those thought to be at high risk of poor outcome by dint of having poor prognostic factors and in others, being more widely applied wherever a matched sibling donor is available. As alloHSCT practice changes, with unrelated donor stem cells being widely available and the use of haploidentical donor cells, or umbilical cord blood becoming more routine, and with the widespread introduction of reduced-intensity conditioning regimens, so its role, which has never been clearly defined by clinical trial, will also change. Its role will also be affected by changes in ALL therapy, for example the use of immunotherapies such as blinatumomab or chimeric antigen receptor (CAR) T cells. As we learn more about predictive factors, develop new predictive biomarkers and have a larger set of therapeutic tools at our disposal, so we will need to alter our focus on who should receive alloHSCT. The application of alloHSCT is one of the most controversial areas of clinical practice in ALL.

Specific approaches to defined clinical populations in the treatment of ALL

Targeted therapies for ALL: Philadelphia chromosome-positive (Ph+) disease

BCR::ABL1+ ALL is identified by the presence of the fusion between the Abelson (*ABL1*) tyrosine kinase gene on chromosome 9 and the breakpoint cluster region (*BCR*) gene on chromosome 22, resulting in a *BCR::ABL1* fusion protein that

is a constitutively active tyrosine kinase. This acquired genetic abnormality is necessary and sufficient for the development of CML (see Chapter 33), but in *BCR::ABL1*+ ALL, other abnormalities, such as deletions within the Ikaros gene, are common and an acute leukaemia occurs *de novo*. In the past, this genetic abnormality conferred the poorest prognosis in ALL. However, the treatment of *BCR::ABL1*+ ALL has been revolutionised by the addition of selective inhibitors of the action of this fusion protein – tyrosine kinase inhibitors, (TKI) – to treatment. These non-chemotherapy agents bind to the kinase domain of *ABL1* and disrupt the ATP binding site. They block the catalytic activity of the kinase and subsequent downstream phosphorylation, essentially 'switching off' a growth signal that is otherwise permanently and erroneously switched on. Although there have been no randomised controlled trials of TKI in *BCR::ABL1*+ ALL, there is no doubt that the overall outcome of therapy is superior with the inclusion of TKIs. Agents that have been evaluated and are licenced for use in ALL include imatinib, nilotinib, dasatinib and ponatinib. Furthermore, many of the more toxic elements of chemotherapy e.g. anthracyclines and L-asparaginase can be omitted without compromising outcome. A higher rate of alloHSCT is typically seen when TKIs are used which may also contribute to the improvement in outcome, although there is now a move to define subsets of patients who may not require alloSCT. There is some evidence that *BCR::ABL1*+ ALL in children can be treated successfully with chemotherapy and TKI combinations alone; in adults, there is no completely compelling evidence to date in the adult setting that *BCR::ABL1*+ ALL can be optimally managed without alloHSCT. ALL generally becomes resistant to TKIs, most often by acquisition of mutations in the kinase domain. Mutational analysis should be obtained in any patient with *BCR::ABL1*+ALL whose disease relapses or becomes resistant to TKI therapy. Many mutations have been mapped and the implications for therapy with various TKI are known. As an example, the occurrence of the T315I mutation confers resistance to most TKIs leaving only ponatinib of clinical utility in this scenario. Recent single-arm studies have shown good outcomes from chemotherapy-free protocols – TKI combined with blinatumomab – in *de novo BCR::ABL1*+ ALL. Longer-term follow-up is needed, but this is likely to represent an important advance.

Ph-like ALL

'Ph-like' or '*BCR::ABL1*-like' ALL is a recently defined entity that encompasses a diverse group of genetic alterations which activate cytokine receptor or kinase signalling. The name derives from the fact that these abnormalities result in a gene expression profile resembling that of *BCR::ABL1*+ALL. This range of genetic lesions typically confers an unsatisfactory response to standard chemotherapy. Although the entity was first identified independently by US and European Groups in 2009, there is still no single diagnostic test or universally agreed approach to uncovering this entity in clinical practice. However, as a practical summary, the majority of patients with this entity will have either (i) JAK-STAT pathway abnormalities, most of which involve cytokine receptor-like factor-2 (*CRLF2)* signalling, through which the JAK-STAT pathway is activated (e.g. *IGH::CRLF2, P2RY8::CRLF2* translocations), or other fusions activating that same pathway including but not limited to those affecting JAK2 and EPOR, the erythropoietin receptor or (ii) ABL-class fusions, including *ABL1*, *ABL2*, *CSF1R*, *LYN* and *PDGF*α::β gene fusions. *BCR::ABL1*-like ALL is not sufficiently common in adults that all patients need to be screened at diagnosis but a suitable screening method should be carried out in any patient with no known primary genetic lesion and a less than optimal response to induction therapy including refractory disease or high-level MRD. This is a very important practical aspect of clinical management as patients with ABL-class fusions often benefit from the addition of TKI to their therapy. At present, there is no evidence for the benefit of JAK-inhibitors in JAK-STAT pathway abnormalities.

Teenagers and young adults

Age is known to be a prognostic factor in ALL. Even in paediatric ALL it is well known that children under the age of 10 years have better outcomes than children over the age of 10 years. These differences are at least partly due to the reduced incidence of good-risk cytogenetics in children over 10 years of age (see Chapter 22). However, it was noticed in the late 1990s that there was a further sharp drop in outcomes for adolescents over 16 years of age. Whilst changing disease biology and patient biology play a role, many national ALL study groups demonstrated a significant impact of the protocol used on long-term outcomes for teenagers and young adults (TYAs) on retrospective data analysis. Traditionally adult protocols have been very transplant-focused, whereas paediatric trials only use first remission transplantation in very high-risk disease. In addition, paediatric protocols historically contained significantly more doses of vincristine and L-asparaginase in induction and consolidation, compared to adult protocols.

In the UK, patients up to the age of 25 years at diagnosis have been treated on national paediatric trials since 2007 (UKALL 2003 and more recently UKALL 2011). With this approach outcomes for TYAs have significantly improved with patients aged 16–25 years now having a predicted long-term survival of 75%, with fewer patients requiring allogeneic stem cell transplantation in first response. However, analysis of outcomes from the UKALL 2003 trial has continued to show an impact of age on outcomes, with adolescents having inferior long-term survival compared to younger children. MRD status post induction and consolidation is the most important predictor of long-term

outcomes in TYAs with ALL. As with older adults, new approaches with the use of novel agents and the selected application of first remission allogeneic transplantation within the context of clinical trials, are required for TYA patients with persistence of high levels of MRD post consolidation therapy.

ALL in older adults

The mean age of adults diagnosed with ALL is 60 years, with an age-specific annual incidence in the UK rising from 0.45/100,000 for adults aged 35–39 to 0.78/100,000 for adults aged 60–64 and 1.2/100,000 for those over 85 years. Despite these figures, patients over the age of 65 years have typically not been included in most large Phase III national trials. The lack of well-designed trials for the older patient with ALL may be one of the reasons why survival in this group has not improved over time.

Importantly, studies have shown that when the effect of cytogenetics on overall survival was accounted for, age was not a significant prognostic factor. This suggests that the worsening prognosis with advancing age in adult ALL could at least in part be a manifestation of the age-related increase in unfavourable cytogenetics.

There is no standard regimen for the older patient worldwide. Data from intensive transplant-based trials show that older patients fared poorly, with 15% long-term survival. Where patients have been deemed ineligible for national trial protocols, local practices have varied widely and treatment is often given with palliative rather than curative intent. Data from the UK National UKALL12 trial reported significantly more infections during induction for patients over the age of 55 years, with chemotherapy doses having to be reduced more commonly in this age group in order to manage toxicity. Infection during induction in this trial was related to poorer outcomes overall. Published data indicate a 3–10 times higher rate of induction deaths in older individuals after treatment for ALL.

Ironically, there has been some progress in improving short-term outcomes for older patients with *BCR::ABL1*+ disease. It is possible to achieve CR with minimal toxicity with the use of a tyrosine kinase inhibitor alone. CR rates of over 90% are reported with imatinib in patients over 55 years of age. When combined with steroid therapy, median survival rates of 20 months from diagnosis are reported, with most patients not requiring any admissions to hospital. The long-term follow-up of these patients suggests ongoing relapses and long-term cures are unlikely with this approach. Balancing toxicity of treatment against potential long-term disease control in older patients is an enormous challenge. Emerging novel agents such as monoclonal antibodies have the potential of adding an independent anti leukaemic effect without the addition of substantial toxicity. The challenge will be how to integrate these novel agents into ALL protocols for older individuals, where a current standard of care is not defined.

Treatment of relapsed/refractory disease

If ALL fails to respond to therapy or relapses in adults, survival has generally been very poor and alloHSCT in second complete remission is the only known curative therapy. Several large studies of patients who relapsed following initial therapy have recently been published. Once relapse has occurred, it is generally the features of the relapsed disease, rather than the disease at original diagnosis which determine outcome. Factors predicting poor outcome from relapse include older age, a short duration of first remission and relapse in the CNS. Salvage therapy in older adults with conventional chemotherapy agents in B-ALL has largely been supplanted by the CD19-targeted bispecific antibody, blinatumomab (CR2 rate approximately 45%) or the CD22-directed antibody-drug conjugate, Inotuzumab ozogamicin (CR2 rate approximately 80%). Both agents are much better tolerated than conventional chemotherapy. Additionally, CD-19 directed chimeric antigen receptor T-cells (CAR-T) are now licenced for treatment of children and adults with relapsed/refractory B-cell ALL.

For relapsed T-ALL, there are fewer directed options: nelarabine is available although is generally used in combination with traditional chemotherapy agents. CAR-T approaches are a more technically difficult option than B-ALL but are under very active pursuit. Other agents under active clinical study include venetoclax combined with navitoclax and the antiCD38 antibody daratumumab Despite the increasing availability of novel agents in this clinical space, the optimal outcome for adult ALL can only be achieved by avoiding relapse. It is highly likely that including novel, non-chemotherapy approaches in de novo disease will facilitate better outcomes and trials are ongoing to determine how best to achieve this.

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CHAPTER 22

Childhood acute lymphoblastic leukaemia

22

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Introduction

Acute Lymphoblastic Leukaemia is the most common form of cancer (25–30%) and predominant subtype of leukaemia (75–80%) in children. However, childhood ALL is not a single disease and has considerable phenotypic and genotypic heterogeneity which is of diagnostic and prognostic importance. Modern protocols take account of this heterogeneity by incorporating complex risk stratification models to determine content and intensity of therapy received by an individual patient. Despite no new drugs being available during that period, treatment outcome has improved substantially in the last four decades such that over 90% of patients can expect to survive without disease in the long term. Translation of recent advances in understanding of the molecular biology of ALL and its influence on phenotype and clinical outcome are identifying specific subgroups that might benefit from targeted therapy.

Epidemiology

The median age at diagnosis for ALL is 13 years and nearly 60% of cases are diagnosed under the age of 25.

There is a sharp peak in ALL incidence amongst 2–3 year olds (>80 per million) which decreases to a rate of 20 per million for 8–10 year olds. The incidence of ALL amongst 2–3 year olds is approximately fourfold greater than that for infants and is nearly tenfold greater than that for 19 year olds. The incidence is higher in boys than in girls (four times for T-cell ALL), except that girls have a slightly higher (1.5 times) incidence of leukaemia in the first year of life. The reported incidence of ALL is higher in Northern and Western Europe, North America and Oceania, and lower in Asia, South America and Africa. In industrialised countries, the incidence is higher amongst children of European descent than amongst those of African descent. For example, the annual rate (per million population) of childhood ALL from birth to 19 years of age is 29.7 in the UK and 11.0 in India; in the USA, it is 32.9 for white people and 14.8 for African–Americans. T-cell ALL and pre-B leukaemia with *TCF3::PBX1* fusion are more prevalent amongst African–American children, who are less likely to have ALL with high hyperdiploidy. The incidence of ALL amongst children younger than 15 years of age has shown a moderate increase in the past 20 years

Aetiology

Genetic factors

Only a small proportion (<5%) of patients with childhood ALL have underlying hereditary genetic abnormalities. Children with Down syndrome have a 10- to 30-fold increased risk of developing ALL. Other genetic disorders associated with an increased incidence of ALL include ataxia telangiectasia, Shwachman-Diamond syndrome and Bloom syndrome. There is no definite evidence of an association between leukaemia and congenital immunodeficiencies such as X-linked agammaglobulinaemia. Patients with Li-Fraumeni syndrome (germline *TP53* mutation)

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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have an increased riks of developing low hypodiploid (30–39 chromosomes) ALL. Recent genomic sequencing of families with an increased frequency of haematological malignancies have identified rare high-penetrance mutations in several genes including *ETV6, PAX5* and *IKZF1* which have been linked to an increased risk of developing childhood ALL. In addition, patients who carry a Robertsonian der(15;21)(q10;q10) translocation have a very high likely of developing ALL with an iAMP21.

Fraternal twins and siblings of affected children are at a twofold to fourfold greater risk of leukaemia during the first decade of life than are unrelated children. In the case of identical twins, when leukaemia occurs in one twin, there is a 20% probability that it will also occur in the other twin, owing to ALL transfer *in utero* via the shared placental circulation. When leukaemia is diagnosed before 1 year of age it almost invariably develops in the other twin, generally within a few months. In identical twins with *KMT2A::AFF1*, the concordance rate is nearly 100%, with a short latency period (weeks to a few months). In contrast, the concordance rate in twins with the *ETV6::RUNX1* fusion or T-cell phenotype is lower and the postnatal latency period longer, in keeping with the requirement for additional genetic events for leukaemic transformation in these subtypes of ALL. High hyperdiploid ALL also appears to arise before birth but requires postnatal events for full malignant transformation. In contrast, *TCF3::PBX1* ALL appears to have a postnatal origin in most cases.

Genome wide association studies (GWAS) have discovered polymorphisms at numerous genomic loci associated with a moderate increased genetic susceptibility to childhood ALL in general or a specific genetic subtype. Germline variants in *ARID5B* have the strongest association with ALL susceptibility across the genome, especially high hyperdiploid ALL, and the loss of Arid5b in mouse leads to significant defects in lymphoid cell development. The lower incidence of B-ALLwith high hyperdiploidy in African–American children in the US may at least partially reflect lower prevalence of the *ARID5B* risk allele. Other polymorphisms in *GATA3* and TP63 have been linked with Philadelphia/*BCR::ABL1*-like and *ETV6::RUNX1* respectively. Polygenic risk analysis indicates that ALL susceptibility increases cumulatively with the number of risk alleles the child carries. Several of the genes implicated in GWAS studies as conferring increased risk of ALL are known to be involved the pathogenesis of ALL; e.g. *IKZF1*, *CDKN2A, CDKN2B*, *CEPEBE*, *ETV6* and *PAX5*. *IKZF1*, an important transcription factor in all lymphoid lineages, is frequently targeted by copy number alterations in ALL blast cells, and *IKZF1* deletion is associated with a poor prognosis. Loss of *CDKN2A/CDKN2B* occurs in up to 40% of B-precursor ALL and is likely to contribute to cell cycle deregulation in leukaemia. *CEBPE* is related specifically to myeloid cell maturation and terminal differentiation, but interchromosomal translocations involving the immunoglobulin heavy locus (*IGH*) and *CEBPE* genes also have been described in childhood ALL. The convergence of germline ALL susceptibility loci and somatic aberrations on genes involved in lymphoid cell development, cell cycle control and tumour suppression reinforces the contribution of these key pathways to leukaemogenesis and also points to the possibility that inherited and acquired genetic variations act synergistically in the development of childhood ALL.

Environmental factors

Ionising radiation and chemical mutagens have been implicated in the induction of leukaemia but clear aetiological factors for ALL cannot be identified in a majority of cases. *In utero* exposure to diagnostic X-rays is associated with a slightly increased risk of ALL, proportional to the number of exposures. The association between leukaemia and maternal exposure to potential mutagens, neonatal administration of vitamin K, parental use of medications and drugs, proximity to electromagnetic fields and exposure to other potential mutagens have been proposed but not proven. In the UK, it has been demonstrated that the geographical incidence of childhood leukaemia shows more clustering than would be expected if it were due to chance variations, which could be due to the geographical distribution of environmental risk factors, including infectious agents.

Some childhood ALL initiating mutations such as *ETV6::RUNX1* fusion originate in utero and are regarded as a first hit. However, these first hits occur at a higher frequency than the incidence of childhood ALL, suggesting the need for additional second hits. The drivers for these second hits are unclear, though infection remains the strongest candidate. Although a time/space cluster of common ALL was reported in Milan with a possible association to Influenza H1N1 infections, rather than being in response to a specific virus, it is more likely that the leukaemic transformation is as a result of an abnormal or dysregulated immune response to one or more common infections (viral or bacterial) in a genetically susceptible individual at a young age when the immune system is less mature.

Further support for a dysregulated immune response to an infection has come from animal studies. Mice with germ line *PAX5* mutations or *ETV6::RUNX1* fusion only develop common ALL when housed in conventional facilities where they are exposed to infectious agents and don't do so if kept in a pathogen-free environment. These genetically predisposed mice also have a distinct gut microbiome compared to wild-type mice. Disrupting the microbiota with antibiotics in these genetically susceptible mice also triggered the development of common ALL even in the absence of infection. These studies suggest that there is a complex interaction between genetic and environmental factors in the development of childhood ALL.

Pathogenesis

As described above, ALL develops through the acquisition of driver mutations that define the characteristics of the disease (Figure 22.1). Typically, an initiating events such as *ETV6::RUNX1* will give rise to a pre-leukaemic clone that expands and, with

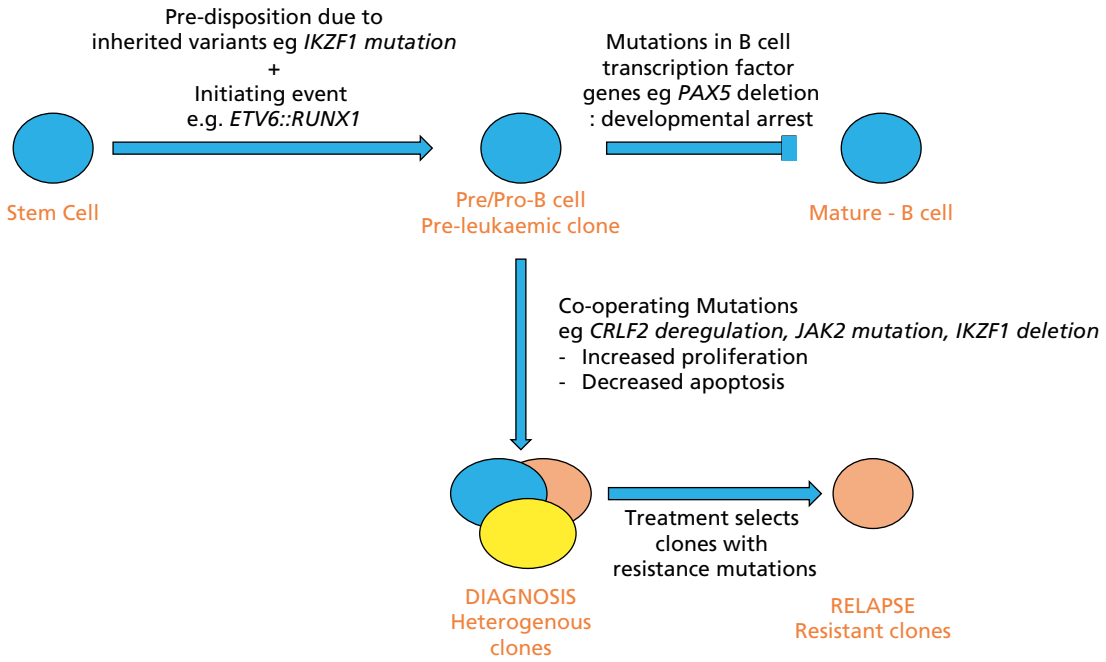

Figure 22.1 Pathogenesis of ALL.

time, acquires secondary co-operating mutations resulting in developmental arrest, increase proliferation and decreased apoptosis. Occasionally, a single genetic event is sufficient to drive the development of the disease as observed in *KMT2A* rearranged infant ALL. The average number of mutations acquired during the pathogenesis is thought to be around six or seven; far fewer than that observed in adult solid tumours. The spectrum of primary or initiating genetic abnormalities ranges from gene fusions to ploidy shifts and complex chromosomal rearrangements (see cytogenetics). Secondary or cooperating mutations are typical micro-deletions or point mutations affecting lymphoid differentiation, cell cycle regulation, cell survival, proliferation and transcriptional cofactors. The majority of patients will harbour a single primary clonal genetic abnormality along with a series of subclonal mutations that give rise to genetic heterogeneity. The spectrum of secondary events is linked, sometimes strongly, to specific primary abnormalities indicating epistatic interactions. Examples include *BCR::ABL1* and *IKZF1* deletion, *ETV6::RUNX1* and loss of the second untranslocated *ETV6* allele and *IGH::DUX4* with *ERG* deletions. The primary abnormality observed at initial diagnosis is always retained at first and subsequent relapses. In contrast, the pattern of secondary abnormalities evolves between each time point often driven by the expansion of treatment-resistant minor subclones. The acquisition of *CDKN2A/B* deletions and RAS pathway mutations are frequent findings. Relapse-specific mutations linked to resistance to certain therapies are increasingly being reported. For example, *NR3C1* mutations linked with glucocorticoid resistance, *NT5C2* mutations and thiopurine resistance, *ABL1* mutations and resistance to tyrosine kinase inhibitors.

Clinical features

Children with ALL frequently present with symptoms and signs of pancytopenia due to marrow failure. Fever, often due to leukaemia rather than infection, is present in 60% of patients. A meta-analysis of more than 3000 children with childhood leukaemia found that more than half presented with at least one of the following five features: palpable liver, palpable spleen, pallor, fever or bruising. Lymphadenopathy was noted in nearly a half of children and musculoskeletal pain was a presenting feature in 43% of cases. Young children with bone pain may present with a limp or refusal to bear weight. Bulky extramedullary disease, especially overt CNS disease, mediastinal mass and renal infiltrates are more common in T-lineage ALL. Mediastinal masses can cause superior vena cava (SVC) syndrome which can present with swelling of the face and upper limbs due to compression of the SVC. CNS involvement is usually lepto-meningeal rather than parenchymal and may present with symptoms of meningism or cranial nerve palsies. Ocular involvement is more common at relapse than first presentation and can affect the retina, ocular nerve, orbit, cornea or anterior chamber with hypopyon. Painless enlargement of the scrotum may be due to a testicular infiltrate or hydrocele resulting from lymphatic obstruction. Rarely, the diagnosis is made in a child who has a blood count for minor, non-specific, symptoms.

Differential diagnosis

Immune thrombocytopenic purpura (ITP) is the most common benign condition to be associated with symptoms that raise concern about leukaemia. Severe skin haemorrhage in an otherwise well child with isolated severe thrombocytopenia and normal blood film without circulating blasts makes it possible to distinguish the condition from leukaemia in a vast majority of cases. The presence of mild anaemia due to nose or gum bleeding and activated lymphocytes on blood film due to a recent viral illness may create diagnostic uncertainty in some cases but it should be possible for an experienced morphologist to exclude the presence of blasts on a blood film without resorting to a bone marrow aspirate.

Pancytopenia due to aplastic anaemia is difficult to distinguish from 'aleukaemic leukaemia' without marrow examination. Rare cases of ALL present with an aplastic marrow without blasts, often accompanying severe sepsis, with spontaneous reconstitution of normal haemopoiesis on recovery from the infection. They represent several weeks to months later with low counts and circulating lymphoblasts. Clonal rearrangements are invariably detected on retrospective examination of the initial marrow using sensitive genetic techniques and it's likely that the 'aplastic' presentation of ALL in such cases is due to severe sepsis causing marrow aplasia followed by spontaneous remission due to a cytokine release phenomenon.

Bone pain, arthralgia and occasionally arthritis may mimic juvenile arthritis in a minority of patients.

Paediatric small round cell tumours that involve the bone marrow, including neuroblastoma, rhabdomyosarcoma and retinoblastoma, may be confused with ALL on marrow morphology but can be easily distinguished on flow cytometry or immune-histochemistry.

Laboratory features

A blood count at diagnosis shows varying degrees of pancytopenia which is more severe in B-lineage than T-lineage ALL. Initial leucocyte counts (Table 22.1) range from 0.5 – $>1000 \times 10^9$/L (median 12 × 10^9/L); they are greater than 10 × 10^9/L in slightly over half of patients and greater than 100×10^9/L in 10–15% of patients. Most patients have circulating blasts, but they may be difficult to detect in those with leucopenia without careful scrutiny of a blood film ('aleukaemic leukaemia'). Reactive hypereosinophilia with pulmonary infiltration and cardiomyopathy is a feature of a rare subtype of B-lineage ALL with t(5;14)(q31;q32)/*IGH::IL3*. Coagulopathy is less common than in acute myeloid leukaemia, usually mild, and not associated with severe haemorrhage except in patients with very high white cell counts who are at risk of CNS haemorrhage due to leukostasis and tumour lysis. Elevated uric acid and phosphorus levels may be seen due to rapid tumour proliferation in patients with high tumour burden. An enlarged kidney can be detected in 30–50% of patients but has no prognostic or therapeutic implications. Raised liver enzymes due to leukaemic infiltration are present in 10–20% of patients, are usually mild and have no important clinical or prognostic consequences. A minority of patients may have cholestatic jaundice due to nodes at the porta hepatis or portal tract infiltration. Abnormalities of the bone, such as metaphyseal banding, periosteal reactions, osteolysis, osteosclerosis or osteopenia, are present in half of the patients. As these changes do not affect treatment and outcome, routine diagnostic imaging studies (except for chest radiography to rule out mediastinal mass) is not necessary.

Leukaemic blast cells are identified morphologically at diagnosis in a cytospin of cerebrospinal fluid (CSF) in approximately one-third of cases, most of whom have no neurological symptoms. Only a minority of such cases (2–3%) fulfil the criteria for CNS leukaemia (CNS3) which is defined by the presence of at least five leucocytes per microlitre of CSF and the detection of leukaemic blast cells, or by the presence of cranial nerve palsy. A recent Scandinavian group (NOPHO) study detected CSF blasts in newly diagnosed ALL patients by cytospin in 13.8% and by flow cytometry in 25.4%. Patients with T-ALL, high count ALL and traumatic lumbar punctures (defined as >10 RBC/μL) were more likely to have blasts detected by flow cytometry. CSF positivity, either detected by a cytospin or by flow cytometry was associated with a higher risk of relapse. Further studies are planned to determine the prognostic value of CSF leukaemia detection by flow cytometry.

A dense infiltrate of lymphoblasts is usually detected in a marrow aspirate. Occasionally, the aspirate may be difficult to obtain and appear hypocellular with sparse blasts due to haemodilution. In such cases, a trephine invariably shows a dense infiltrate but in rare cases with 'aplastic' presentation (see differential diagnosis) may also be severely hypocellular. Morphological analysis of leukaemic cells distinguishes three subtypes of ALL (L1, L2 and L3) according to the French–American–British (FAB) classification. Unlike in AML, the FAB classification has no prognostic or therapeutic relevance in ALL and, along with cytochemical staining, has been replaced by immunophenotypic and genotypic diagnostic investigation and classification.

Immunophenotypic classification

Early pre-B ALL

Leukaemic blast cells of early pre-B ALL resemble normal marrow B-cell precursors. The leukaemic cells always express CD19 and almost all cases have cytoplasmic CD22 and CD79α; surface CD22 expression is also evident in most cases. CD10 and terminal deoxynucleotidyltransferase (TdT) are expressed in 90% of

Table 22.1 Characteristics and 10-year survival rates for 3113 patients in UKALL 2003 (10/2003–06/2011).

			Relapse rate (RR)			Event free survival (EFS)			Overall survival (OS)		
Variables		**Number of subjects in the whole cohort**	**Events at 10 yrs**	**RR (95%CI)**	***P* value**	**Events at 10 yrs**	**EFS (95%CI)**	***P* value**	**Events at 10 yrs**	**OS (95%CI)**	***P* value**
Overall	*N* = 3113	*n* (%)	310	10.8 (9.7–12.0)		421	85.8 (84.5–87)		308	89.6 (88.4–90.6)	
Sex	Male	1767 (57%)	204	12.4 (10.9–14.1)	<0.05	266	84.3 (82.4–85.9)	0.07	195	88.4 (86.8–89.9)	<0.05
	Female	1346 (43%)	106	8.6 (7.2–10.3)		155	87.8 (85.8–89.5)		113	91.1 (89.4–92.6)	
Age	<10 yrs	2279 (73%)	190	8.9 (7.8–10.2)	<0.001	254	88.4 (87.0–89.7)	<0.001	163	92.5 (91.3–93.5)	<0.001
	10–15 yrs	607 (20%)	76	14.2 (11.4–17.5)		107	80.6 (76.9–83.8)		88	84.2 (80.7–87.1)	
	16–24 yrs	227 (7%)	44	21.4 (16.4–27.7)		60	72.4 (65.9–77.8)		57	73.9 (67.4–79.4)	
WCC($x10^9$/L)	<50	2472 (78%)	213	9.4 (8.3–10.7)		288	87.5 (86.0–88.8)	<0.001	204	91.2 (89.9–92.3)	
	>=50	685 (22%)	97	15.6 (13.0–18.7)	<0.001	133	79.7 (76.4–82.6)		104	84.0 (80.9–86.7)	<0.001
CNS	CNS1	2919 (94%)	279	10.3 (9.2–11.6)		378	86.4 (85.0–87.6)		273	90.0 (89.0–91.2)	
	CNS2	160 (5%)	23	15.4 (10.5–22.3)		34	78.4 (71.1–84.1)	<0.001	27	82.4 (75.3–87.6)	<0.001
	CNS3	34 (1%)	8	24.9 (13.3–43.6)	<0.001	9	72.7 (54.1–84.8)		8	76.5 (58.4–87.5)	
Down syndrome	Yes	85 (3%)	19	31.3 (20.8–17.0)		34	53.6 (41.0–64.6)	<0.001	31	62.6 (51.1–72.1)	<0.001
	No	3028 (97%)	291	10.3 (9.2–11.5)		387	86.7 (85.4–87.9)		277	90.3 (89.2–91.4)	
NCI Risk	Standard	1812 (58%)	137	8.0 (6.8–9.4)	<0.001	181	85.6 (88.1–91.0)	<0.001	113	93.4 (92.2–94.5)	<0.001
	High	1301 (42%)	173	14.8 (12.8–17.0)		240	80.3 (77.9–82.5)		195	84.1 (81.9–86.1)	
MRD level	Negative	1616 (60%)	97	6.5 (5.4–7.9)	<0.001	130	91.4 (89.8–92.7)	<0.001	66	95.5 (94.3–96.5)	<0.001
	Positive	1062 (40%)	169	17.0 (14.8–19.5)		225	78.2 (75.5–80.6)		167	83.6 (81.1–85.7)	
Immunophenotype	B-ALL	2727 (88%)	255	10.1 (9.0–11.4)		344	86.7 (85.3–87.9)	<0.001	250	90.3 (89.1–91.4)	
	T-ALL	386 (12%)	55	15.3 (11.9–19.4)	<0.01	77	79.7 (75.2–83.4)		58	84.6 (80.5–87.9)	<0.01

cases, and CD34 in more than 75% of cases. The CD20 antigen is present on a minor proportion of blast cells in half of cases but its intensity might increase during treatment. Early pre-B ALL cells lack expression of surface and cytoplasmic immunoglobulins.

ALL with *KMT2A* fusions typically have an early pre-B (pro) ALL phenotype with distinctive features such as expression of CD15, CD65 and chondroitin protoglycan sulfate and absence of CD10. High hyperdiploidy is typically associated with weak or undetectable CD45 expression and *ETV6::RUNX1* with aberrant CD13/33 expression.

Pre-B ALL

The pre-B immunophenotype is defined by the accumulation of cytoplasmic immunoglobulin μ heavy chains with no detectable surface immunoglobulins and is found in approximately 20–25% of cases. Leukaemic cells that express both cytoplasmic and surface immunoglobulin μ heavy chains without κ or λ light chains, a rare finding, have been designated transitional pre-B ALL. Pre-B ALL expresses CD19, CD22 and CD79α and, usually, CD10 and TdT but only two-thirds express CD34. In many cases of pre-B ALL, surface CD20 is absent or is weakly expressed. ZAP-70 expression is more prevalent in cases with a pre-B phenotype. Between 20 and 25% of pre-B ALL cases have *TCF3::PBX1* fusion.

(Mature) B-cell ALL

In 2–4% of childhood ALL cases, cells express surface immunoglobulin μ heavy chains plus either κ or λ light chains. Commonly, cells have L3 morphology according to the FAB classification and express CD19, CD22, CD20 and frequently CD10; CD34 is negative. The less common subtype of B-cell ALL is characterised by blast cells with L1 or L2 morphology, and expression of TdT and/or CD34. These patients should be treated for stage IV B non-Hodgkin's Lymphoma (Burkitt's lymphoma) if they have re-arrangement of the *MYC* gene.

T-lineage ALL

Around 15% of children and young people have T-lineage ALL, but the incidence is lower in younger children and increases with age. T-lineage ALL cells express CD7 and CD3, the latter most commonly only in the cytoplasm. Other markers commonly expressed include CD2, CD5 and TdT; CD1a, surface CD3, CD4 and CD8 are detected in fewer than 45% of cases. HLA-DR expression is uncommon, and 40–45% of cases are positive for CD10 and/or CD21. CD79α is also weakly expressed in approximately one-third of cases.

T-lineage ALL has been divided into three stages of immunophenotypic differentiation: early ($CD7^+$, $cCD3^+$, surface CD3−, CD4− and CD8−), mid or common ($cCD3^+$, surface CD3−, $CD4^+$, $CD8^+$ and $CD1a^+$) and late (surface $CD3^+$, CD1a− and either $CD4^+$ or $CD8^+$). However, many cases have immunophenotypic patterns that do not fit these thymic maturation stages. T-cell receptor (TCR) proteins are heterogeneously expressed in T-lineage ALL. In approximately two-thirds of cases, membrane CD3 and TCR proteins are absent. In half of these cases, however, TCR proteins (TCR-β, TCR-α, or both) are present in the cell cytoplasm. Most cases with membrane CD3 and TCR chains express the αβ form of the TCR, whereas a minority express TCR γδ proteins.

A subtype of T-ALL, named early thymic precursor (ETP) ALL has been recently identified, which exhibits the gene expression profile of normal ETP cells, a population of recent immigrants from the bone marrow to the thymus that retains multilineage differentiation potential. The phenotype is characterised by positive CD7 and cCD3, negative CD1a and CD8 expression, weak or absent CD5 expression and expression of at least one stem cell or myeloid-associated antigen (e.g. CD34, CD117, CD13, CD33 and CD11b). ETP ALL was reported as having a dismal outcome in initial reports but a more recent UK report shows that it's outcome is only slightly worse than for non-ETP ALL

Cytogenetic and molecular classification

High Hyperdiploidy (51–65 chromosomes)

High hyperdiploidy (HeH) is the most common primary genetic abnormality observed in 35% children with ALL. The frequency decreases with age and HeH is observed in less than 20% of adolescents and young adults. It is defined by the non-random gain of whole chromosomes typically trisomies of 4, 6, 10, 14, 17 and 18, tetrasomy of 21 and additional copies of chromosome X. Numerous studies have shown that patients with HeH have a better outcome than their age-matched counterparts, which is possibly due to methotrexate sensitivity. Several studies have reported differential outcome according to the pattern of trisomies and some study groups use specific combinations to define patients with the lowest risk of relapse (e.g. triple trisomy (+4, +10 and +17), UKALL HeH good profile based on the trisomic status of chromosomes 5, 17, 18 and 20)

Near-haploidy (<30 chromosomes)

Near-haploidy is a rare finding in paediatric ALL accounting for <1% cases but is associated with a very high rate of relapse. The pattern of chromosome loss is non-random. Chromosome 21 is never lost, monosomy of chromosome 14 is rare and the sex chromosomes are usually retained. Near-haploid leukaemia clones often evolve by chromosome doubling which can cause a diagnostic dilemma because the modal chromosome number of the doubled component overlaps with high hyperdiploidy. SNP array analysis is the ideal technique for resolving this dilemma.

Low hypodiploidy (30–39 chromosomes)

Low hypodioloidy is another rare (1%) ploidy subgroup associated with a very high risk of relapse. The frequency increases with age and it occurs in 10% of older adults with ALL. It is distinct from near-haploidy because 90% cases harbour a *TP53* mutation that is germline in ~50% cases. Hence genetic testing and counselling for Li-Fraumeni Syndrome is advised when this subtype is detected. As with near-haploidy, the pattern of chromosome loss is non-random and chromosome doubling is a common feature that can cause diagnostic issues.

ETV6::RUNX1 fusion

The chromosomal translocation, t(12;21)(p13;q22), results in the chimeric fusion product *ETV6::RUNX1* (formerly *TEL::AML1*) and is the most prevalent translocation in paediatric ALL (~25% BCP-ALL) but it is rare amongst adults. Unlike many chromosomal translocations, it is cytogenetically cryptic, and hence detection requires FISH or molecular testing. Virtually all major clinical trial groups around the world have reported that children with *ETV6::RUNX1* fusion enjoy excellent overall survival with very low rates of relapses. There is some evidence to suggest that the favourable outcome of *ETV6::RUNX1* ALL is related to optimal asparaginase therapy.

TCF3::PBX1 fusion

Approximately 20–25% of pre-B ALL cases (5% B-ALL cases) have *TCF3::PBX1* fusion resulting from t(1;19)(q23;p13) or the unbalanced form [der(19)t(1;19)]. The translocation juxtaposes the *TCF3* (formerly *E2A*) gene on chromosome 19 and the *PBX1* gene on chromosome 1. The resulting *E2A::PBX1* fusion protein contains the transcriptional activation domains of E2A linked to the DNA-binding domain of PBX1 and the encoded protein inappropriately activates the transcription of genes normally regulated by *PBX1*. With contemporary treatment protocols, patients with this leukaemia subtype have excellent response to initial therapy and a favourable overall outcome. Importantly, ~5% of cytogenetically visible t(1;19)(q23;p13) translocations involved other genes, hence FISH or molecular confirmation of *TCF3::PBX1* fusion is important.

TCF3::HLF fusion

Another *E2A* fusion gene is created by t(17;19)(q22;p13), in which *E2A* is fused to the gene that encodes the transcription factor hepatic leukaemia factor (*HLF*). Patients with ALL and this gene fusion frequently present with hypercalcaemia and coagulopathy. This gene fusion is extremely rare (<0.1%) but is almost universally fatal with very few known survivors.

Fusions involving *KMT2A* (formerly *MLL*)

Structural alterations involving band q23 on chromosome 11 are the most frequent cytogenetic abnormality in infant ALL and occurs in 1–2% of older children in both B-cell and T-cell disease. The target is the *KMT2A* gene formerly called *MLL* (for mixed-lineage leukaemia). The most common *KMT2A* fusion results from the t(4;11)(q21;q23) and produces a chimeric protein that contains the N-terminal portion of *KMT2A* linked to the C-terminal portion of *AFF1*. Numerous other *KMT2A* fusions (>50) have been reported with the C-terminal portion of different partner genes. The *KMT2A* gene encodes a DNA-binding protein that regulates the expression of many genes including multiple *HOX* genes. *KMT2A* is crucial for embryonic development and haemopoiesis, and *KMT2A* fusion proteins can transform haemopoietic cells into leukaemia-initiating cells. Treatment outcome of patients with *KMT2A* fusions differs by age group, with infants having the worst outcome, but they are always considered high risk. Infant ALL with the *KMT2A::AFF1* gene fusion also has a high prevalence of immature, non-productive and/or oligoclonal antigen-receptor gene rearrangements.

BCR::ABL1 fusion

The t(9;22)(q34;q11) translocation encodes a chimeric gene consisting of the 5′ portion of *BCR* fused to the 3′ portion of *ABL1*, whereby N-terminal sequences of *ABL1* are replaced by *BCR* sequences. In ALL, breaks tend to occur in the minor breakpoint cluster regions, forming a 190-kDa *BCR::ABL*. This alteration results in a constitutively active ABL tyrosine kinase that induces aberrant signalling and activates multiple cellular pathways. Genome-wide analysis of *BCR::ABL1* ALL samples revealed deletions of *IKZF1* in >60% of cases whereas deletions were not found in chronic-phase CML samples. Moreover, deletion of *IKZF1* appeared to be a lesion acquired at the time of transformation from CML to lymphoid blast crisis. *BCR::ABL1* ALL is refractory to standard chemotherapy but outcomes have improved significantly in recent studies in which a tyrosine kinase inhibitor (TKI) such as Imatinib was given in combination with chemotherapy throughout a 2-year treatment programme. Thus, first remission allogeneic stem cell transplant is no longer indicated for the vast majority of patients (nearly 90%) who obtain a good MRD response after 12 weeks of chemotherapy plus TKI. Patients with resistance to first-generation TKI agents may respond to second and third-generation TKIs such as nilotinib, dasatinib and bosutinib.

Intrachromosomal amplification of chromosome 21q (iAMP21)

This recurrent chromosomal abnormality defines a distinct genetic subgroup of childhood ALL. They have a common/pre-B immunophenotype, are significantly older (median 9 years) and

have a lower white cell count (median 3.9) compared to children without this abnormality. When treated with standard-risk chemotherapy iAMP21 patients had a very relapse risk (>80% at 10 years) However, when treated with high-risk chemotherapy (as per UKALL2003) the risk of relapse reduces to <20% at 10 years.

BCR::ABL1-like ALL

Around 15% of B-ALL cases exhibit a gene expression profile similar to that of *BCR::ABL1* ALL but do not have the fusion. They commonly harbour *IKZF1* deletions and have a poor outcome. Next-generation sequencing, including transcriptome and whole-genome sequencing, has shown that these cases harbour a diverse range of rearrangements, deletions and sequence mutations often activating cytokine receptor and kinase signalling. The majority, but not all, *BCR::ABL1*-like cases can be classified as ABL-class fusions or JAK-STAT abnormalities.

JAK-STAT abnormalities

This genetic subgroup comprises genomic rearrangements whose downstream consequence is the constitutive activation of the JAK-STAT pathway. The rearrangements *P2RY8::CRLF2*, *IGH::CRLF2*, *JAK2* fusions and *EPOR* deregulation. *EPOR* deregulation occurs via point mutations or more rarely *IGH::EPOR* fusion. *JAK2* fusions comprise the 3′ end of *JAK2* fusing with variable 5′ partner genes (e.g. *PAX5* and *BCR*). *IKZF1* deletions are common in this subgroup and *JAK2* mutations frequently accompany *CRLF2* rearrangements. The frequency of this subtype is 5–10% B-ALL cases but >50% Down syndrome patients with ALL will harbour a *CRLF2* rearrangement, often *P2RY8::CRLF2*. The outcome of patients with a JAK-STAT abnormality is not well established. While some study groups have reported a poor outcome the relapse risk is not as high as observed for patients with classical high-risk genetics (e.g. *KMT2A* fusions, iAMP21 and *BCR::ABL1*).

ABL-class fusions

This subgroup of gene fusions include fusions involving the 3′ end of *ABL1 (*except *BCR::ABL1)*, *ABL2*, *PDGFRB*, *CSF1R* and, more rarely, *PDGFRA*. The resulting fusion has similar functional consequences to the canonical *BCR::ABL1* fusion, i.e. deregulation of tyrosine kinase activity. The 5′ partner genes are highly variable but include *EBF1, ETV6, NUP214* and *SSBP2*. The most prevalent fusion is *EBF1::PDGFRB* accounting for up to 50% cases. Studies have found that patients with an ABL-class fusion have very high levels of MRD post induction and a significantly higher relapse risk when treated with standard chemotherapy. Although predominantly found in BCP-ALL, studies have identified cases with T-ALL especially *NUP214::ABL1*. There is good *in vitro*, *in vivo*, *ex vivo* and clinical evidence that patients harbouring an ABL-class fusion are sensitive to tyrosine kinase inhibitors (TKI), e.g. imatinib and dasatinib. This evidence includes reports of patients with refractory disease at EOI achieving complete remission after treatment with a TKI. Several clinical study groups now give patients with an ABL-class fusion adjuvant TKI therapy.

IGH::DUX4 fusions

Overexpression of *DUX4* results from *IGH::DUX4* fusion or more rarely *ERG::DUX4* fusion and occurs in ~5% B-ALL cases. *DUX4* over-expression is accompanied by transcriptional deregulation of *ERG*, usually via deletion, resulting in loss of function of ERG, a transcriptional regulator, required for normal haematopoietic stem cell function. Patients with a *DUX4* fusions have a very good outcome with several study groups reported relapse rates as low of 2–3%. *DUX4* is located on the long arm of chromosome 4 in the highly repetitive subtelomeric region. This location and presence of multiple copies make the detection of *IGH::DUX4* extremely challenging. Accurate detection requires careful analysis of whole genome or RNA sequencing.

Other genetic abnormalities in B-ALL

Genomic analysis including whole genome sequencing and RNA sequencing have identified and continue to identify novel genetic abnormalities. The frequency and prognosis of these abnormalities have not been established and will require large comprehensive prospective studies. These abnormalities are invariably novel gene fusions or clusters of abnormality driving distinct expression profiles. *ZNF384* gene fusions occur in ~3% B-ALL cases but up to 40% patients diagnosed with mixed phenotype acute leukaemia (MPAL). They are associated with low CD10 expression and aberrant CD13/33 expression. The most common partner genes are *EP300, TCF3* and *TAF15*. *MEF2D* fusions (usually with *BCL9* or *HNRNPUL1*) account for a similar proportion of B-ALL cases and also have low CD10 expression. These cases frequently have high expression of CD38 and results in increased *MEF2D* expression and in turn activation of the histone deacetylase, *HDAC9*. Genes from the CEPB family (e.g. *CEPBA*, *CEPBE*, *CEPBG*, etc.) and *ID4* are over-expressed via juxtaposition to the IGH enhancer. These cases are rare overall but tend to be older children or young adults. The *ETV6* and *PAX5* genes are well-known to be involved in ALL development via specific gene fusions (e.g. *ETV6::RUNX1* and *PAX5::JAK2*) and deletions (see below). Transcriptomic and genomic studies have identified distinct subgroups termed *ETV6::RUNX1*-like and PAX5alt which encompasses a range of abnormalities centred on these two genes and include variants fusions and mutations.

Copy number alterations

Microdeletions of key B-cell development and cell cycle genes are the most prevalent secondary abnormalities in ALL. One or more deletions affecting *EBF1, IKZF1, CDKN2A/B, PAX5,*

ETV6, BTG1, RB1 or the pseudo-autosomal region (PAR1) occurs in 60% of children with B-ALL. Extensive risk profiling by the UKALL study group revealed that patients without any deletion affecting these genes/regions or isolated deletions of *ETV6, PAX5, BTG1* or *ETV6* deletions with a single additional deletion of *BTG1*, *PAX5*, *CDKN2A/B* comprised a good risk group. This UKALL-CNA good risk group was enriched for cases with *ETV6::RUNX1* and high hyperdiploidy but also defined as a good risk amongst other B-ALL cases. In contrast, *IKZF1* deletions are associated with a poor outcome overall but their effect is context-dependent. Patients with an IKZF1plus profile (*IKZF1* deletion plus concomitant deletion of *CDKN2A/B*, *PAX5* or PAR1 in the absence of *ERG* deletion) and high MRD at the end of induction have a very poor outcome.

Genetic abnormalities in T-cell ALL

The genetic and genomic landscape of T-ALL is largely distinct from that observed B-ALL. The exceptions include *KMT2A* fusion, ABL-class fusions and *CDKN2A/B* deletions. There are at least six distinct genetic subgroups of T-ALL defined by type A abnormalities that block T-cell differentiation. The subgroups are driven by deregulation of *TAL1*, *TLX1*, *TLX3*, *NKX2-1*, *HOXA* or *LMO2/LYL1*. In addition, to type A abnormalities all patients will harbour one or more type B abnormality which are mostly deletion and mutations affecting proliferation and cell cycle control. The most frequently affected genes include *CDKN2A/B*, *NOTCH1*, *FBXW7*, *PTEN*, *KRAS*, *NRAS* and *PHF6*. None of the T-ALL genetic abnormalities predict outcome to the extent that treatment should be stratified on the basis of specific abnormalities.

Prognostic factors

Presenting features

Age, presenting white cell count and immunophenotype are independent predictors of relapse risk in childhood ALL. Although the association of outcome with age and presenting white cell count is linear, current risk stratification models use a threshold for both variables to define standard and high-risk groups. For example, the National Cancer Institute (NCI) criteria employed by the UKALL and US Children's Oncology groups categorises patients below 10 years with white cell count less than 50×10^9/L as standard risk (60% of patients) and older patients or those with a higher white cell count as high risk.

Historically, the outcome for boys was worse compared with girls but the difference is not evident in some contemporary studies. In some, but not all, US studies, patients of African–American and Hispanic origin had a significantly worse outcome.

Primary chromosomal and genomic abnormalities are strong independent prognostic factors that are frequently used to risk-stratify patients. Patients with *ETV6::RUNX1*, high hyperdiploidy, *IGH::DUX4* and *TCF3::PBX1* have all been associated with good response to standard chemotherapy and multiple studies have reported 5-year EFS rates exceeding 90%. Patients with *ETV6::RUNX1* and high hyperdiploidy who have other good risk features such as low WCC, young age or low MRD post induction are frequently eligible for treatment with reduced or lower intensity chemotherapy. In contrast, patients with *BCR::ABL1*, *KMT2A* fusions, near-haploidy, low hypodiploidy, iAMP21, *TCF3::HLF* and ABL-class fusions are deemed high-risk abnormalities and have invariably been linked with poor survival in multiple studies. Patients with iAMP21 have improved outcome when treated with high-risk chemotherapy but survival is still <85%. Patients with *BCR::ABL1* or ABL-class fusion have improved outcome when treated with a TKI. Whilst many of the other primary genetic subtypes listed above have been associated with linked outcome, the evidence to date is insufficient to justify altering therapy. Several secondary abnormalities, usually CNA profiles, are emerging as robust prognostic factors. However, there is less consistency in approach than for primary genetic abnormalities. Several studies have focussed on *IKZF1* deletions as a high-risk biomarker but use different definitions (deletion versus IKZF1plus profile) and have chosen different interventions. One study has opted to incorporate the UKALL-CNA good risk profile to identify patients who have intermediate-risk MRD and a low risk of relapse.

Early response

Risk stratification based on clinical criteria, though, is relatively non-specific. For example, a high-risk group with a 5-year EFS of around 50% defined by age, gender and presenting WCC identifies only 20% of patients destined for relapse, with the majority of relapses still arising out of the remaining, apparently, low-risk patients. Speed of response in the first few weeks of treatment is a powerful determinant of outcome. Morphological slow early response, measured as slow clearance of blood or marrow blasts after a week or two of induction therapy, is associated with a relatively high risk of relapse and around 1% of patients who are not in complete remission at the end of induction (induction failures) have a poor outcome. However, over 60% of relapses arise from the group with a rapid morphological response in the first 2 weeks of therapy. Measurement of Minimal Residual Disease (MRD) offers a much more sensitive and specific means of distinguishing between patients who will and will not relapse.

Minimal residual disease

The bone marrow of patients in morphological complete remission may contain up to 10^{10} residual leukaemic cells. This submicroscopic level of leukaemia (Minimal Residual Disease, MRD) can be detected using sensitive molecular or flow cytometric methods. Molecular quantitation of MRD using

Real-Time Quantitative PCR of antigen receptor gene rearrangement (Immunoglobulin and T-cell receptor) is the method of choice in the UK and Europe. Each leukaemic clone carries a unique molecular signature of rearranged antigen receptor genes which can be detected at diagnosis using appropriate PCR primers and sequenced. A patient-specific probe designed against the junctional region of the rearranged gene is then used to track the leukaemic clone in remission samples. Flow cytometric methods utilise combinations of cell surface antigens uniquely expressed on leukaemic cells (Leukaemia Associated Immunophenotype, LAIP) to detect MRD.

Several large studies have revealed that the positive and negative predictive value of a particular MRD result depends on the sensitivity of the technique used to measure it, the time point at which it is measured, and the treatment received by the patient before and after the point of assessment. Despite differences in these variables amongst published studies, there is sufficient consensus in the results relating to certain clinical endpoints. Around 50% of patients become MRD negative for two clonal markers by the end of induction (EOI) and have a <5% risk of relapse. Patients with detectable MRD at the end of induction subdivide into two groups on follow-up. In around 95% of these patients (45% of all patients) the MRD level falls below 0.5% by the end of consolidation therapy. This group has a 20% relapse risk and benefits from further intensification of therapy as demonstrated in UKALL 2003. The remaining 5% (1% of all patients) have persistent MRD ≥0.5% at post induction follow-up and an associated >80% risk of relapse. The optimal therapy for this MRD very high-risk group is uncertain and many groups consider them eligible for experimental therapy followed by first remission allogeneic stem cell transplant. The kinetics of MRD clearance is slower in T-lineage compared with B-lineage ALL and the prognostic outlook for a given detectable MRD level is worse at earlier time points for B-lineage and later time points for T lineage.

Integrated risk factor analysis

Historically, risk factors in ALL were usually binary and applied sequentially without integration. Analysis of UKALL2003 data revealed that end of induction (EOI) MRD was log-normally distributed and MRD distributions of patients with distinct genetic subtypes were different (Figure 22.2). Patients with good-risk cytogenetics demonstrated the fastest disease clearance, whereas patients with high-risk genetics and T-cell disease responded more slowly. The risk of relapse was correlated with MRD kinetics, and each log reduction in disease level reduced the risk of relapse by 20%. Although the risk of relapse was directly proportional to the MRD level within each genetic risk group, the absolute relapse rate associated with a specific MRD value or category varied significantly by genetic subtype. Hence adopting genetic-specific MRD thresholds improves relapse prediction. This concept has been integrated in the ALLTogether1 trial risk stratification algorithm where three separate MRD thresholds are used for different genetic subtypes to selected good-risk patients for the intermediate-low-risk treatment group (Figure 22.3) intermediate-risk MRD patients. Future risk algorithms are likely to build upon these findings to develop and implement fully integrated models. The UKALL study group has developed and validated a prognostic index that integrates genetics, white cell count and end of induction MRD to assign patient-specific risk scores on a continuous scale (Figure 22.4). One of the major advantages of such an approach is the ability to tailor the number, size and outcome of risk groups to the treatment options or randomisation under investigation.

Pharmacogenetic variables

Mutations and polymorphisms of genes encoding several drug-metabolising enzymes have been associated with response to therapy. The best-known are thiopurine *S*-methyltransferase (TPMT) gene, which encodes the enzyme that catalyses mercaptopurine to non-cytotoxic methyl mercaptopurine metabolites and Nudix Hydrolase 15 (*NUDT15*) gene which degrades oxidised purine nucleoside triphosphates (e.g. 8-oxo-dGTP) by dephosphorylation to prevent incorporation into DNA. Approximately 1 in 300 patients have an inherited homozygous deficiency of TPMT which results in all the administered dose of mercaptopurine (a pro-drug) being converted to cytotoxic thioguanine nucleotides (TGNs).

Patients heterozygous for *TPMT* and/or *NUDT15* risk variants in general tolerate a dose of 6MP of 50–60 mg/m^2, and some even 75 mg/m^2. Thus, pre-emptive dose reductions are not recommended for these patients. Patients who are homozygous for *TPMT* low activity alleles or homozygous for *NUDT15* low activity alleles should as a starting dose receive 6 MP 5 mg/m^2/day (i.e. 5–10% of standard dose) to avoid marrow aplasia. If tolerated the dose can then carefully be increased. Recently, low activity polymorphisms have been described in the *NUDT15* gene that encodes the Nudix hydrolase 15 protein which hydrolyses the thiopurine active metabolites 6-Thio-GTP and 6- thio-DGTP. Low-activity genotype variants of *NUDT15* are inherited as monogenic autosomal codominant traits associated with increased risk of myelosuppression, especially in the homozygous state. Inherited *TPMT* deficiency is primarily seen in Europeans and Africans, whereas risk alleles in *NUDT15* explain most of thiopurine-related myelosuppression in Asians and Hispanics with native American ancestry. *NUDT15* variants are rare amongst Europeans (<1.0%) and even rarer in Africans. However, a recent study in a London population has indicated a higher frequency of *NUDT15* variants in self-identified British Caucasian and mixed-race individuals in addition to those of Asian ancestry. Hence all UK patients are genotyped for the common *NUDT15* and *TPMT* variants in an accredited laboratory.

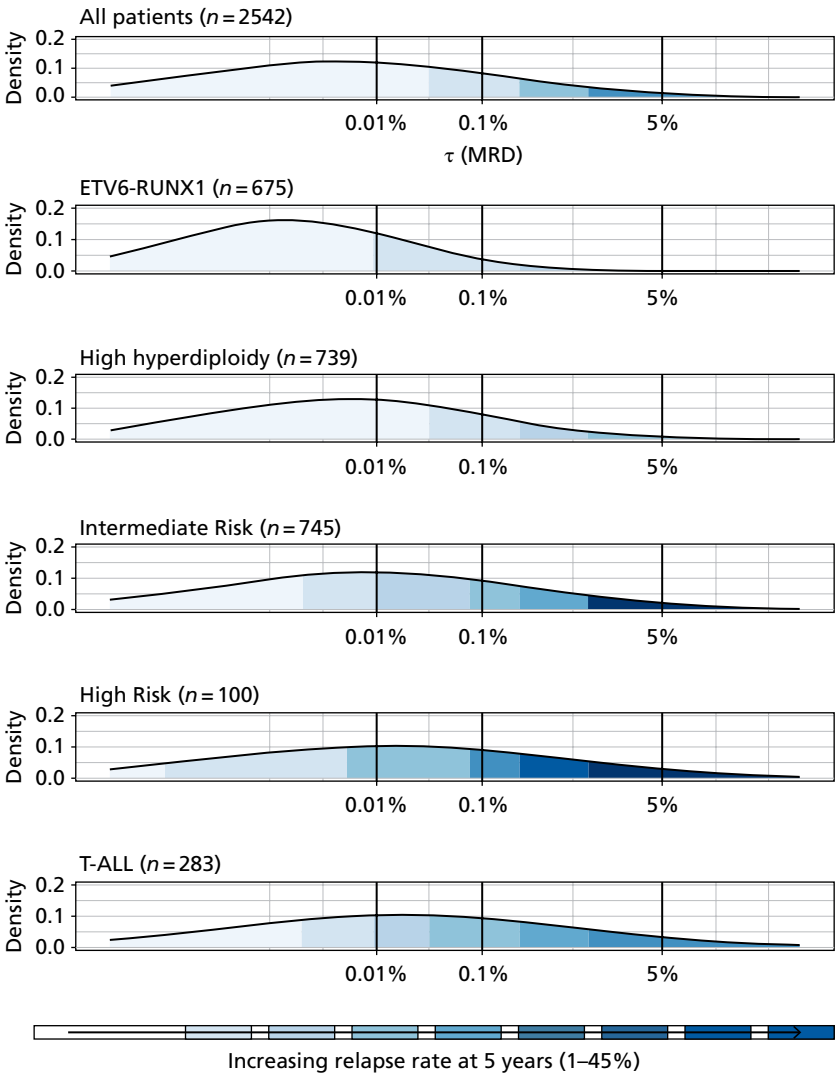

Figure 22.2 Relationship between minimal residual disease (MRD) and relapse risk by different genetic subtypes. Absolute MRD values were log-transformed [τ(MRD)] and each panel shows a smoothed density distribution for cases in a particular genetic subtype. The darker shading indicates high risk of relapse.

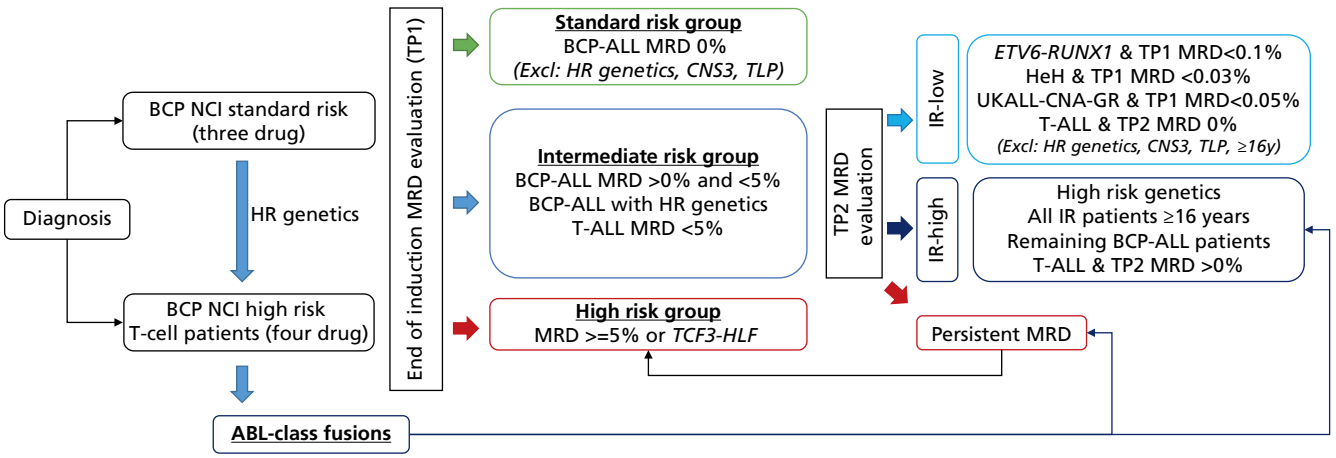

Figure 22.3 ALLTogether1 trial risk stratification algorithm. BCP, B-cell precursor; MRD 0%: undetectable MRD by IG/TCR PCR; HeH, high hyperdiploidy; High-risk genetics: *KMT2A* fusions, near haploidy, low hypodiploidy and iAMP21. ABL-class: *ABL1, ABL2, PDGFRB, CSF1R* fusions. UKALL Copy Number Alteration (CNA)Good Risk profile: no deletion of *IKZF1, CDKN2A/B, PAR1, BTG1, EBF1, PAX5, ETV6, RB1*; isolated deletions of *ETV6, PAX5, BTG1, ETV6* deletions with a single additional deletion of *BTG1, PAX5, CDKN2A/B*. *Courtesy of Anthony Moorman, John Moppett and Mats Heyman on behalf of the ALLTogether consortium.*

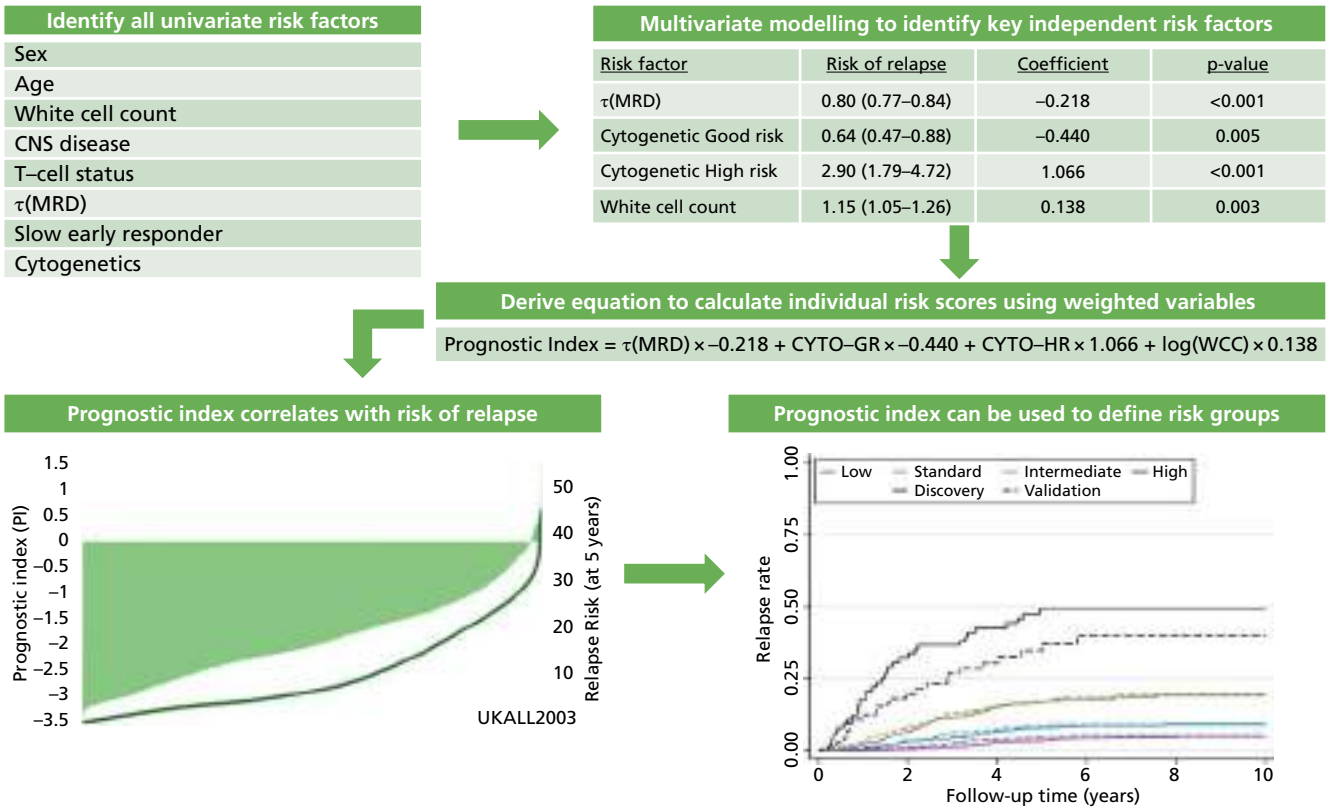

Figure 22.4 Development of the UKALL prognostic index using discovery (UKALL2003) and validation (DCOG10, NOPHO2008 and COALL97/03/09) cohorts totalling >4500 cases.

Treatment

Drugs and protocols

Eleven categories of chemotherapeutic drugs form the mainstay of childhood ALL therapy (Table 22.2). Current front-line treatment protocols contain varying combinations of these eight drugs given over a 2–3-year period. A typical treatment regimen consists of induction, pre-emptive CNS-directed, intensification and maintenance (also called Continuing) therapy courses. Induction and intensification therapy are designed for rapid debulking of tumour load during the first few months of therapy, while maintenance therapy, administered over the remaining 2–3 years, is aimed at clearing MRD. Systemic therapy has variable penetration across the blood–brain barrier and, therefore, is insufficient to prevent isolated CNS relapse, the risk of which is substantially reduced by pre-emptive CNS-directed therapy.

Historical background

Studies conducted by collaborative groups in many different countries have contributed to the current generally gratifying outcome of treatment of childhood ALL. The first attempt at a cure was pioneered in the mid-sixties by the St. Jude's group in Memphis, USA, who showed that durable remissions could be achieved in roughly 30–40% of patients with a combination chemotherapy protocol, which they called total therapy, containing remission induction, pre-emptive Central Nervous System (CNS) therapy and prolonged continuation therapy. While other groups tried to optimise this basic template by doing randomised studies of various components within it, the Berlin-Frankfurt-Munster (BFM) group took the second major step forward in the late 1970s, documenting that long-term remission rates could be improved to 60–70% by intensified induction and consolidation therapy. Others, including the MRC in the UK, subsequently confirmed the benefits of intensified therapy even using different combinations and schedules of intensification from the original BFM model. Subsequent UK studies have investigated alternatives to cranial radiotherapy for CNS-directed treatment (UKALLXI) and compared the efficacy and toxicity of dexamethasone with prednisolone and thioguanine with mercaptopurine (ALL97). Cranial radiotherapy and HD MTX reduce the risk of CNS relapse compared with prolonged IT MTX but are associated with equivalent overall EFS due to an increase in non-CNS relapses. Hence, presently in the UK, cranial radiotherapy is restricted to patients with overt CNS disease

Table 22.2 Drugs used in the treatment of childhood ALL.

Drug Class	Mechanism of Cytotoxicity	Route(s) of administration	Part in treatment protocol	Significant Toxicities	
				Acute	Late
Vinca Alkaloids	Disruption of mitotic spindle	Intravenous bolus	Throughout	Neurotoxicity Constipation Vesicant	Neurotoxicity
Glucocorticoids	Apoptosis by binding to intracellular Steroid Receptors	Oral and intravenous	Throughout	Hyperglycaemia Weight gain Mood alteration Hypertension	Osteoporosis Obesity Avascular Necrosis of Bone
L-Asparaginase	Depletion of L-Asparagine	Sub-cutaneous or Intra-muscular	Induction and Intensification	Hypersensitivity Thrombosis Pancreatitis Hyperlipidaemia Neurotoxicity	None?
Anthracyclines	DNA intercalation Inhibition of Topo-isomerase II function	Intravenous infusion	Induction and Intensification	Vesicant Myelosuppression	Cardiotoxicity
Alkylating agents (Cyclophosphamide)	Interstrand DNA cross-links	Intravenous bolus or infusion	Intensification	Myelosuppression Haemorrhagic cystitis	Secondary leukaemia Infertility
Methotrexate	Anti-folate	Oral, intravenous and intra-thecal	Maintenance CNS-directed therapy	Mucositis Nephrotoxicity (High Dose) Hepatotoxicity	Osteopenia Neurotoxicity
Thiopurines (Mercaptopurine and Thioguanine)	Incorporation of thioguanine nucleotides into newly synthesised DNA	Oral.	Maintenance	Hepatotoxicity Myelosuppression	Hepatic?
Epipodophyllotoxins (Etoposide and Teniposide)	Interferes with topo II religation of double-stranded DNA breaks	Intravenous infusion	Intensification	Myelosuppression	Secondary leukaemia
Cytarabine	Inhibits DNA polymerase	Intravenous, Sub-cutaneous or intra-thecal	Intensification	Hypersensitivity Myelosuppression Central neurotoxicity	Neurotoxicity
Blinatumomab	Bispecific antibody. Cytotoxicity through T-cell engagement with CD19 positive cells	Continuous intravenous infusion	Relapse, MRD positivity	Cytokine release syndrome Neurotoxicity Myelosuppression	B cell aplasia
Inotuzumab	Antibody targeted calicheamin mediated cytotoxicity of CD22 positive cells	Intravenous	Relapse	Hepatoxicity Myelosuppression	Veno-occlusive disease post SCT
Tisagenlecleucel (CD 19 directed CART)	Cytoxicity of CD19 cells by re-engineered chimaeric antigen T cells expressing CD19 directed antibody	Intravenous	Relapse, Primary refractory	Cytokine release syndrome Neurotoxicity Myelosuppression	B cell Aplasia

(CNS3) at presentation who have persistent CSF blasts after two intrathecal methotrexate injections. Dexamethasone is more effective than prednisolone in reducing the risk of systemic and CNS relapses in several studies. Thioguanine is more effective than mercaptopurine at preventing CNS relapses, especially in younger boys, but is associated with an increased risk of death in remission and sinusoidal occlusion syndrome or veno-occlusive disease (SOS/VOD) of the liver. A proportion of patients with the latter toxicity have chronic portal hypertension due to periportal liver fibrosis.

Current UK strategy

UKALL 2019 guidelines

When no clinical trial is available for childhood ALL, the usual approach is to follow the standard treatment arms of the most recent randomised trial which in the UK was UKALL 2011. This treatment regimen designated UKALL 2019 guidelines is described below. The guidelines are designed for all patients from age 1 (first birthday) to 24 years 364 days (at time of diagnosis) with a first diagnosis of acute lymphoblastic leukaemia or lymphoblastic lymphoma (T-NHL or SmIg negative precursor B-NHL) diagnosed using standard criteria.

Treatment

Patients receive 1 of 3 escalating intensity treatment regimens depending on their clinical and MRD risk group. Initial treatment allocation is on the basis of clinical risk criteria with treatment post induction being determined by MRD response.

Induction and consolidation

NCI standard risk precursor B cell precursor ALL standard risk patients receive a three drug induction containing vincristine, steroids and asparaginase for 4 weeks (Regimen A) and NCI high-risk patients receive in addition Daunorubicin (Regimen B). All patients receive three doses of intrathecal methotrexate (IT MTX) in induction with patients who have blasts in their cerebrospinal fluid at diagnosis receiving an additional two doses. All patients with T cell ALL (independent of NCI risk) will receive a Regimen B induction. A small number of NCI Standard Risk BCP ALL patients initially treated according to Regimen A induction will subsequently be found to have high-risk cytogenetics (*KMT2A* fusions, near haploidy, low hypodiploidy, iAMP21 and *TCF3::HLF*) and should transfer to Regimen C induction (four drug induction) on day 15 of induction.

For consolidation, Regimen A patients who are MRD low-risk receive daily oral mercaptopurine and three doses of weekly intrathecal methotrexate. Regimen B patients who are MRD low-risk receive in addition 4 weeks of cyclophosphamide and cytarabine (Berlin Frankfurt Munster (BFM) consolidation). Patients with high-risk cytogenetics or who are MRD positive at end of induction transfer to Regimen C. Regimen C patients receive an additional four doses of vincristine and two doses of pegylated asparaginase during the BFM consolidation course (called Augmented BFM Consolidation).

Primary refractory ALL

B-lineage patients with induction failure (defined at end of induction MRD>5%) receive a Regimen C consolidation. Depending on age, phenotype and response patients may receive licensed autologous CART (if mid-consolidation MRD>1%) or be recruited to a CART trial (if NCI high risk and end of consolidation MRD>0.01%). Patients not eligible for CART are recommended Blinatumomab (bispecific CD19/3 targeting antibody) as a bridge to transplant. Patients not responding to Blinatumomab receive intensive chemotherapy such as FLA-Ida as bridge to transplant. Younger children (<16 years) with good response to consolidation continue on Regimen C.

Based on the results of COGAALL0434 study, T-ALL patients with induction failure receive Regimen C consolidation + Nelarabine as bridge to transplant. Those refractory to this combination either receive intensive chemotherapy such as FLA-Ida or experimental treatment including Daratumumab or Venetoclax/Navitoclax.

Interim maintenance and delayed Intensification

Following consolidation, patients receive 2 months of interim maintenance prior to a single delayed intensification course. MRD low-risk (Regimen A/B) patients receive oral mercaptopurine and methotrexate with monthly vincristine and steroid pulses during interim maintenance. Interim maintenance for MRD intermediate-risk patients (Regimen C) consists of escalating doses of intravenous methotrexate without folinic acid rescue, vincristine and pegylated asparaginase.

All patients receive a single delayed intensification. For MRD low-risk (Regimen A/B) patients, this consists of a single dose of pegylated asparaginase at day 4 and vincristine, dexamethasone, doxorubicin for 3 weeks followed by cyclophosphamide and cytarabine as given during the BFM consolidation course. MRD intermediate (Regimen C) patients receive in addition two doses of vincristine and one dose of pegylated asparaginase.

Continuation therapy

Regardless of clinical and MRD risk group, all patients receive oral mercaptopurine and methotrexate, monthly vincristine and steroid pulses and 3 monthly intrathecal methotrexate. Boys receive treatment for 3 years and girls for 2 years from the start of interim maintenance.

Steroid and asparaginase formulations, doses and schedules

All patients received dexamethasone 6 mg/m^2 with a 10 mg ceiling dose during induction and maintenance courses. In delayed intensification courses, all patients receive dexamethasone at 10 mg/m^2

(without a cap) for 14 days in a week on, week off schedule. All patients receive pegylated asparaginase 1000 units/m^2/dose given intramuscularly throughout treatment. Regimen A/B patients receive three doses (two in induction and one in delayed intensification course) and Regimen C patients eight doses (additional two in interim maintenance and delayed intensification courses).

Central nervous system (CNS) directed therapy

Patients with ≥5 leucocytes/microlitre and blasts in a diagnostic cerebrospinal fluid (CSF) sample with <10/microl red cells (CNS-3) receive an extra two IT MTXs in induction. Patients with traumatic lumbar puncture and blasts in the CSF as well as those with <5 leukocytes/microlitre which were blasts (CNS-2) also receive an extra two IT MTXs during induction. All other patients received intrathecal methotrexate as described above. Regimen C patients receive Capizzi intravenous methotrexate at doses <500 mg/m^2 without folinic acid rescue. Thus no patients receive cranial radiotherapy or high-dose methotrexate during first-line treatment

Allogeneic haemopoietic stem cell transplantation

Less than 5% of patients are eligible first remission transplant, primarily for primary refractory disease once they have achieved an optimal MRD response (<0.01%).

ALLTogether1 protocol

In 2014, an international consortium of study groups came together to develop a common protocol for newly diagnosed children and young adults with acute lymphoblastic leukaemia (ALL). The consortium includes study groups from the UK (UKALL), The Nordic countries plus Lithuania and Estonia (NOPHO), Germany (CoALL), Belgium (BSPHO), Portugal (SHOP), France (SFCE), Ireland (PHOAI) and the Netherlands (DCOG). The new trials that commenced in summer 2020 will recruit approximately 1400 patients annually and employ a new risk stratification (see above). The trial will address randomised questions about de-escalation of treatment for low-risk groups and use of novel agents (Inotuzumab, TKIs and CART) for higher-risk patients.

Current outcomes

Given the above treatment, the 5-year event-free survival (EFS) of over 3000 children and young people (ages 1–25 years) recruited to UKALL 2003 (2003–2011) was 87.2%, which compares favourably with outcomes reported from contemporary trials by other groups (Tables 22.1 and 22.3). A low incidence of isolated CNS relapse (1.9%) was observed, equivalent to that observed in studies in which a significantly higher proportion of patients received cranial irradiation. Randomised interventions within the trial demonstrated that treatment can be de-escalated without compromising survival in MRD low-risk patients, and augmentation of post-remission therapy reduces relapse risk for patients with detectable MRD at the end of induction. Thus, in the current UK trial, post-remission treatment intensity is stratified by MRD response at the end of induction and recovery from consolidation.

The improved outcomes reported in UKALL 2003 and other recent trials have been obtained in the absence of new drugs for the treatment of ALL for over 40 years. The use of dexamethasone and pegylated asparaginase throughout treatment is likely to be an important contributor to the improvement seen in UKALL 2003. Several large randomised clinical trials have demonstrated the improved efficacy of dexamethasone in preventing systemic and CNS relapse compared with prednisolone. Pegylated asparaginase has better pharmacokinetic and pharmacodynamic properties than the native formulation and a lower risk of hypersensitivity reactions on re-exposure. The successor study to UKALL 2003, UKALL 2011 study recruited patients between 2011 and 2018 and a preliminary analysis has shown similar outcomes to UKALL 2003.

Treatment of distinct subgroups

ABL-class fusions

A subset of patients with *BCR::ABL1*-like ALL harbour an ABL-class fusion. Patients with refractory disease on the UKALL2003 trial harboured a high frequency of ABL-class fusions (10%) Specifically *EBF1::PDFGRB* patients had high levels of end of induction MRD and a high rate of relapse An analysis of 13 patients with an ABL-class fusion on UKALL2011 treated with imatinib in first remission demonstrated a superior event free survival compared to contemporary controls ($n = 8$) not treated with a tyrosine kinase inhibitor. As such, all patients ABL class fusion are treated with imatinib (340 mg/m^2/day) on day 15 of induction which is then given continuously. They are also transferred to Regimen C with further treatment allocation dependent on end consolidation MRD.

Young people (age 16–25 years)

A decade ago, retrospective comparisons demonstrated a consistent and large EFS or OS advantage for young people with ALL treated according to paediatric protocols compared with adult protocols. The reasons for this were unclear but possibly included physician experience and compliance, patient compliance, supportive care and specific aspects of protocol design. In particular, early dose intensification of chemotherapy, higher cumulative doses of steroids, vincristine and L-asparaginase and less frequent use of alkylating agents, anthracyclines, high dose cytarabine and allogeneic stem cell transplant (with the associated higher treatment-related mortality) in paediatric protocols.

Table 22.3 Outcomes with contemporary childhood ALL protocols.

Trial	Group	Region	Years	Subgroup (n)	EFS (yrs)	OS (yrs)
Several	COG	US, Canada, Australia, New Zealand	2006–10	All patients (8090)	N/A	91.5% (5-yr)
				B-ALL (7397)	N/A	92.0% (5-yr)
				T-ALL (676)	N/A	90.6% (5-yr)
Total 16 (age 0–18)	SJCRH	US	2007–17	All patients (598)	88.2% (5-yr)	94.1% (5-yr)
				B-ALL (494)	89.8% (5-yr)	95.6% (5-yr)
				T-ALL (104)	81.0% (5-yr)	87.0% (5-yr)
05001/11001	DFCI	US, Canada	2000–15	All patients (551) (05001)	85.0% (5-yr)	91.0% (5-yr)
				B-ALL (678)	87.0% (5-yr)	93% (5-yr)
				T-ALL (123)	81% (5-yr)	90% (5-yr)
AIEOP-BFM 2000	BFM	Western Europe	2000–06	All patients	N/A	N/A
				B-ALL (4016)	80.4% (7-yr)	91.8% (7-yr)
				T-ALL (464)	75.9% (7-yr)	80.7% (7-yr)
ALL-10	DCOG	Netherlands	2004–12	All patients (778)	87% (5-yr)	91.9% (5-yr)
				B-ALL (661)	88.1% (5-yr)	93.3
				T-ALL (116)	80% (5-yr)	84.4
UKALL 2011 (age 1–25)	MRC/NCRI	UK	2012–2022	All (2800)	87% (4-yr)	92%
				B (320)	89% (4-yr)	93%
				T (2480)	82% (4-yr)	87%

AIEOP-BFM, Association of Italian Paediatric Oncology and Berlin Frankfurt-Munster; COG, Children's Oncology Group; DFCI, Dana Farber Cancer Institute Consortium; SJCRH, St. Jude Children's Research Hospital; MRC, Medical Research Council; NCRI, National Cancer Research Institute.
* infants <1 year old excluded where possible.

The results of these retrospective comparisons have been validated in prospective trials that recruited 16–25 year old patients to a paediatric protocol, such as UKALL 2003, in which that age group had a 5-year EFS of 75%.

Infants

Acute lymphoblastic leukaemia (ALL) in infants under 1 year of age is rare and biologically different from ALL in childhood. Infant ALL is characterised by a high frequency of *KMT2A* gene rearrangements, a very immature B-cell phenotype (proB ALL), expression of myeloid markers, lack of CD10 expression, presentation with a high tumour load and a poor outcome. *KMT2A* rearrangement status, presenting white blood cell count (WBC), age at diagnosis and prednisone response are independent prognostic factors and a model comprising age, *KMT2A* status and WBC stratifies patients into three risk groups with distinct 4-year EFS (Low: *KMT2A* germ line, EFS 74%; Intermediate: *KMT2A* fusion, WBC < 300 or age < 6 months, EFS 43% and High: *KMT2A* fusion and age < 6 months and WBC > 300, EFS 18%) in the Interfant99 international trial. Its successor, Interfant 06, combined this risk stratification algorithm and MRD response to select patients for first remission allogeneic transplant. The trial also tested 'myeloid' versus lymphoid consolidation but the former was not found to be of benefit except in a subgroup with expression of myeloid markers and MRD > 0.05% at the end of induction. Unfortunately, the overall outcome for *KMT2A* fusion patients was poor even with myeloid consolidation. A recent small Japanese study has reported better outcomes using a chemotherapy dose-intensive approach combined with CR1 HSCT in a higher proportion of patients than Interfant06. Molecular investigations, pre-clinical studies and early phase clinical studies indicate that epigenetic modifiers and immune-based approaches might be effective in *KMT2A* fusion cases. The UK is piloting an approach of replacing toxic consolidation therapy with Blinatumomab which will form the basis for a non-randomised question in Interfant21. Recent studies have found that *KMT2A* germ line infant ALL patients have genetic abnormalities similar to those seen in older patients, including *NUTM1* and *PAX5* fusions. The former is associated with an excellent prognosis and, in the UK, *KMT2A* germ line patients have been treated the same as older children since Interfant06 closed.

Down syndrome

Children and young people with DS have a 10–20-fold increased risk of developing ALL compared to those without DS. This increased risk is limited to the first three decades of life but with a notable absence of ALL in those under a year of age. T cell ALL is rare in DS, as is the presence of CNS disease at diagnosis. ALL associated with DS (DS-ALL) has a distinct frequency of genetic changes from ALL seen in the non-DS population. Recurring cytogenetic abnormalities conferring either a favourable or poor prognosis are less common and *CRLF2* rearrangements (especially *P2RY8::CRLF2*) are present in nearly 50%. Secondary events including *IKZF1* deletions and *JAK2* mutations are also common. DS-ALL historically had an inferior survival due to a combination of increased relapse risk and high treatment-related mortality (TRM) (21 versus 3% for non-DS-ALL on UKALL2003). TRM occurred in all treatment phases in the DS-ALL cohort unlike the non-DS cohort where TRM occurred predominantly in induction. As a result, several modifications were made from July 2009 in UKALL2003 and for the successor trial UKALL 2011 trial including better supportive care and de-escalation of treatment. As a result, TRM has reduced to 2% (similar to non-DS) without an increase in relapse risk. In the forthcoming ALLTogether1 Study (see below), DS-ALL patients with detectable end of induction MRD Blinatumomab will be replacing 2 consolidation chemotherapy blocks to further reduce toxicity while maintaining efficacy.

Relapse

Relapse may occur during or after treatment, the latter usually within 2 years after cessation of therapy but occasionally later (relapses occurring as late as 10 years after diagnosis have been reported but rarely). The bone marrow remains the most common site of relapse, but the proportion of patients with relapse involving an extra-medullary site, particularly the CNS, has steadily increased as systemic first-line therapy has become more effective. Even when extra-medullary relapse appears to be an isolated event, it is often associated with submicroscopic residual disease in the bone marrow. Although the clonal populations at relapse may not genetically identical to the dominant population at diagnosis, sensitive PCR techniques reveal that they are present as minor subclones that have been selected due to their relative resistance to chemotherapy or through acquisition of epigenetic changes.

The chances of cure after relapse are related primarily to timing of relapse in relation to first diagnosis, immunophenotype, genetic risk group and site. Adverse risk factors include short initial remission, high-risk genetics (see above), T-cell immunophenotype and poor response to relapse therapy. The outcome for relapse patients has not significantly improved in the last 20 years, particularly for high-risk relapses who had dismal outcomes with 5-year EFS and OS of 24 and 26% respectively in the UKALLR3 trial and subsequent UK audit of relapse outcomes. Furthermore, intensive chemotherapy with or without CR2 HSCT exposes patients to a high risk of treatment-related mortality (10–15%) and morbidity including late neuro-cognitive side effects of cranial radiotherapy and TBI. Hence the approach in the UK since 2018 has been to replace the toxic elements of treatment with immune therapy to reduce toxicity whilst maintaining efficacy. Preliminary analysis of this approach is showing promise and is supported by results of randomised studies showing improved disease-free survival and reduced toxicity with Blinatumomab consolidation compared with intensive chemotherapy in high-risk relapses. Other options are the anti-CD22/calicheamycin conjugate antibody Inotuzumab which has been shown to induce MRD-negative remissions in 80% of relapse patients. CD19-directed CAR-T cell therapy (Kymriah) is licensed for primary refractory and post-transplant or refractory relapse and offers a chance of cure to patients who have failed all other treatment including HSCT. Real-world experience using a stringent definition of events to include B cell recovery and use of further therapy shows a 1-year EFS of 34%. Ongoing studies of autologous and allogeneic CART cells are addressing their role in first-line and first-relapse settings including those with isolated CNS relapse. There are also studies of CART cell therapy for relapse T-cell ALL which are showing promise as are those targeting multiple antigens.

Early and late toxicity

Modern intensive therapy is associated with a small risk of mortality (1% in induction, 2% in CR) and significant acute and late morbidity (Table 22.4). A majority of the latter are attributable to cranial radiotherapy and there has been a gratifying reduction in the number of patients with endocrine, growth and neuropsychological problems since it was replaced by alternative approaches to prevention of CNS relapse. Dexamethasone is more toxic than prednisolone and likely to be a significant contributor to the risk of serious bacterial and fungal infection. In a recently concluded European study, AIEOP-BFM 2000, there were twice as many induction deaths amongst patients in the dexamethasone arm compared with the prednisolone arm and other studies have reported a similar experience. Infection remains the biggest cause of TRM. An analysis of infection-related mortality (IRM) on UKALL 2003 demonstrated that the incidence of IRM was 2.4% accounting for 30% of trial deaths and 64% of TRM deaths. The risk for IRM as a proportion of TRM was greater in induction than other phases (77 versus 56%; $P = 0.02$). Sixty-eight percent of cases were associated with bacterial infection (64% Gram-negative) and 20% with fungal infection. Down syndrome was the most significant risk factor for IRM (odds ratio 12.08). The excessive IRM in Down syndrome has now reduced substantially following the amendments made in the subsequent study UKALL2011 (see section on Down syndrome for further details).

Table 22.4 UKALL 2003 serious adverse events.

Risk group	All patients (%)	Reg A (%)	Reg B (%)	Reg C (%)	*p*-value (Reg C vs A/B)
Any infection	307 (13.3)	134 (11.1)	98 (17.5)	75 (14.0)	0.63
Fungal infection	81 (3.5)	25 (2.1)	35 (6.2)	21 (3.9)	0.58
Encephalopathy (excluding seizure)	164 (7.1)	43 (3.6)	58 (10.4)	63 (11.7)	**<0.0001**
Asparaginase Hypersensitivity	47 (2.0)	4 (0.3)	3 (0.5)	40 (7.4)	**<0.0001**
Pancreatitis	33 (1.4)	9 (0.7)	8 (1.4)	16 (3.0)	**0.0006**
Osteonecrosis	88 (3.8)	9 (0.7)	52 (9.3)	27 (5.0)	**0.097**
Thrombosis	51 (2.2)	20 (1.7)	15 (2.7)	16 (3.0)	0.17
CNS Thrombosis	31 (1.3)	12 (1.0)	10 (1.8)	9 (1.67)	0.45
Number in risk groups	2300	1203	560	537	

There is also concern that dexamethasone is associated with a higher risk of osteonecrosis than prednisolone, but that has not been a universal experience. Toxicity attributable to asparaginase occurs in a significant proportion and is dependent on the cumulative exposure to Asparaginase. In the NOPHO 2008 study, a randomised intensification of PEG-asparaginase did not improve EFS but was associated with almost a doubling of serious adverse events such as hypersensitivity, pancreatitis, thrombosis and osteonecrosis) compared with the control arm.

Treatment in a resource poor setting

The drugs, staffing and infrastructure required to deliver the treatment described above are very expensive with an estimated UK cost of £50,000/case for first-line therapy and over £1million for CART therapy using number needed to treat cost analysis. Over 80% of new cases of ALL will arise in countries that cannot afford this cost. Thus, there is an urgent need for studies to test more affordable treatment protocols that can be delivered within a resource-poor setting. The challenge of delivering optimal care to such a large number of patients in a resource-poor setting has been met by clinicians in those countries through a combination of adaptation and innovation, including use of generic alternatives for expensive drugs, although the latter bring with them issues of quality control particularly of biological agents. For a number of reasons, apart from lack of resources, outcomes are worse compared with the West, even within a tertiary care setting. A lower incidence of good-risk karyotypes (*ETV6::RUNX1*, high hyperdiploidy), malnutrition, partial compliance and poor general hygiene conspire to increase the risk of relapse and infection-related deaths. There are no easy solutions to these problems. Simple flow cytometric algorithms for measurement of MRD may be applicable in this setting and could provide a relatively cheap method to identify low-risk patients who can be cured without post-remission intensification therapy, thus avoiding infection-related deaths in this group. Learning from the experience of tuberculosis, pilot programmes should test the feasibility of directly observed treatment to improve compliance with maintenance chemotherapy. Universal access to treatment though remains the single most difficult challenge in this setting. As these nations' overall wealth increases, only a massive investment in public health infrastructure will address this challenge.

Future strategies and conclusions

Given the good results of current protocols, future studies of childhood ALL therapy are faced with the law of diminishing returns. However, there remains a substantial minority of patients with primary refractory disease (around 2%) or early relapse (10%) who cannot be cured with current treatment, including Haemopoietic Stem Cell Transplant. These patients can be identified in first CR as having persistent high-level MRD during the first 20 weeks of treatment. Intervention with novel agents (monoclonal antibodies, tyrosine kinase inhibitors, Nelarabine, Clofarabine and CART cells) with or without HSCT early in first CR might offer some of these patients a cure.

More importantly, attention is being focussed on reducing treatment-related deaths which make up an increasing proportion of treatment failures. Identification of groups at high risk of toxicity (e.g. Down syndrome) and pharmacogenomic expression profiling will guide targeted supportive care and individualised drug dosing to reduce toxic deaths. There is evidence that gene expression profiling of leukaemic blasts can predict *in vitro* and *in vivo* chemosensitivity and treatment in future could be customised to a patient's pharmacogenomic and leukaemia gene expression profiles. A recent study testing Blinatumomab to replace toxic chemotherapy and HSCT has shown that such a 'de-escalation' strategies can maintain and improve EFS by reducing both relapse rates and treatment-related mortality and morbidity. Translation of recent advances in understanding the molecular biology of ALL and its influence on phenotype and clinical outcome are defining specific

subgroups that might benefit from targeted therapy, such as TKIs for patients with ABL-class fusions. Lastly, international collaboration, as highlighted by the INTERFANT and ALLTogether consortia, will need to increase to properly investigate new treatment strategies and the biological determinants of treatment response in increasingly small subcohorts of patients.

Acknowledgements

This version of the chapter retains some of the content of a previous version written by Dr. Ching-Hon Pui and Dr. Dario Campana.

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CHAPTER 23

Chronic lymphocytic leukaemia and other chronic B-cell lymphoproliferative disorders

23

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Classification

Chronic B-cell lymphoproliferative disorders include several entities arising from mature B lymphocytes, which involve primarily the blood, bone marrow and lymphoid organs such as the lymph nodes and the spleen. A constant finding in all these entities is the presence in peripheral blood of leukaemic cells in various degrees. Some of these conditions can be considered primary leukaemias. Others represent the leukaemic phase of B-cell lymphomas and are largely discussed in Chapter 26.

Table 23.1 lists chronic B-cell lymphoproliferative disorders with leukaemic expression as discussed in this chapter.

General aspects of diagnostic methodology

The first diagnostic step is to verify the presence of a non-transient blood lymphocyte count $>5 \times 10^9$/L since many benign conditions may cause moderate and temporary lymphocytosis. Next is the examination of a peripheral blood smear by an experienced haemato-pathologist. This is followed by the study of the immunophenotype (B or T) and determining whether the process is clonal or polyclonal. Once a monoclonal B-cell proliferation is confirmed, the diagnosis needs to be further clarified by using a panel of monoclonal antibodies and genetic markers (Table 23.2).

In those cases in which the absolute lymphocyte count is high ($>5 \times 10^9$/L) and the blood film and immunophenotype show unequivocal features of chronic lymphocytic leukaemia (CLL), lymph node histology is not required. If the diagnosis of lymphoma is a possibility, it is essential to perform a lymph node biopsy. Bone marrow biopsy can confirm a diagnosis of CLL or lymphoma and inform about the mechanism of anaemia or thrombocytopenia. For disorders with an enlarged spleen, such as hairy-cell leukaemia (HCL), splenic marginal zone lymphoma (SMZL) and the non-nodal, leukaemic mantle-cell lymphoma (MCL), spleen histology may be necessary to make the diagnosis. Fluorescence *in situ* hybridisation (FISH) allows the assessment of cytogenetic abnormalities in interphase cells. There is no specific genetic abnormality in CLL but FISH is important for excluding MCL with t(11;14)(q13;q32) and follicular lymphoma with t(14;18)(q32;q21). Next-generation sequencing techniques are unfolding the molecular heterogeneity of B-cell chronic lymphoproliferative disorders but are not employed in routine diagnosis. Finally, whenever possible, biological material not used for diagnostic purposes should be preserved, which can be useful for both research purposes and further studies of clinical interest for the patient.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 23.1 B-cell chronic lymphoproliferative disorders with leukaemic expression.

Chronic B-cell lymphoproliferative disorders
Chronic lymphocytic leukaemia
Small lymphocytlc lymphoma
Monoclonal B-cell lymphocytosis
B-cell lymphomas/leukaemias
Splenic marginal zone lymphoma
Hairy-cell leukaemia
Splenic B-cell lymphoma/leukaemia with prominent nucleoli (Hairy-cell leukaemia variant)
Mantle-cell lymphoma (non-nodal, leukaemic)
Splenic diffuse red pulp small B-cell lymphoma
Lymphoplasmacytic lymphoma
Waldenström macroglobulinaemia

Chronic lymphocytic leukaemia

Definition

CLL is a biologically and clinically heterogeneous disorder caused by the relentless accumulation in bone marrow, peripheral blood and lymphoid tissues of monoclonal B lymphocytes with a distinct immunophenotype (i.e. SmIg^{weak}, CD5^{+}, CD19^{+}, CD23^{+} and CD200^{+}) and a wide range of immune defects. CLL and small lymphocytic lymphoma (SLL) are the same mature B-cell neoplasm that differs in the extent to which the tumour involves blood (CLL) or lymphoid tissue (SLL). Within the SLL/CLL spectrum, about 10% of cases present as SLL and 90% as CLL. In many instances, SLL evolves to CLL over time. The median survival of patients with CLL is >10 years, but the individual prognosis is highly variable, ranging from a few

Table 23.2 Immunophenotypic and genetic markers in chronic B-cell lymphoproliferative disorders.

	SmIg	CD20	CD5	CD10	CD23	CD11C	CD103	CD200	OTHERS
CLL/SLL (MBL)	−/+	−/+	+	−	+	−/+	−	+	
SMZL	+	+	−/+	−	−	−/+	−	+	BCL-2(+); del(7q32) *CD43(+), CD180(−), NOTCH-2 mutations*
HCL	+	+	−	−	−	+	+	+	*BRAF* V600E mutations Annexin D1 (+) Cyclin D1 (weak) CD25(+), CD123(+) Mutated *IGHV*
Non-nodal MCL	+	+	+	−	−	−	−	−	t(11;14)(q13;q32); Cycline D1(+), SOX11(−), CD200 (−) CCND2 translocation (IGK, IGL) Mutated *IGHV*
LPL	+	+	−	−	−		−	−	*MYD88* L265P mutation, *CXCR4* mutations. IgM M component
SLLPN	+	+	−	−	+	+	+	−	CD11C (+), CD123 (+), ANNEXIN A1 (−), Cyclin D1 (−). No *BRAF* V600E mutation *MAP2K1* mutations
SDRPL	+	+	−	−	−	+	+		ANNEXIN D1 (−) Cyclin D3 (+) *CCND3* mutations

CLL, chronic lymphocytic leukaemia; SLL, small lymphocytic lymphoma; HCL, hairy-cell leukaemia; LPL, lymphoplasmacytic lymphoma; Non-nodal MCL, Non-nodal, leukaemic mantle-cell lymphoma; SDRPL, splenic diffuse red pulp lymphoma; SLLPN, splenic lymphoma/leukaemia with prominent nucleoli; SMZL, splenic marginal zone lymphoma.

years to a normal lifespan. Despite important progress in its therapy, in most instances, CLL remains an incurable disease.

Epidemiology

CLL is the most frequent form of leukaemia in Western countries. The disease predominates in males (1.5–2:1) and the median age of patients at diagnosis is around 70 years. The incidence of CLL dramatically increases with age, reaching >20 cases per 100,000/year in individuals older than 70. In Europe, the incidence rate for all subtypes of leukaemia is 7.2 per 100,000/year, of which 34% of cases are CLL, translating to approximately 12,500 new CLL cases annually. Each year, CLL is responsible for approximately 6000 deaths across Europe. In the United States, the age-adjusted incidence rate is 4.2 cases per 100,000/year.

Genetic predisposition

The relative risk of developing CLL in patients' relatives is three to 8 times higher than in the normal population. The molecular basis of familial CLL is currently being unravelled by genome-wide association studies, including both families and sporadic cases. Familial CLL often co-segregates with other B-cell disorders, mainly lymphomas. Also, the prevalence of monoclonal B-cell lymphocytosis (MBL), a situation which may precede CLL, in the normal population is 3–12%, while in first-degree relatives of patients with CLL is 13–18%. While in Western countries CLL accounts for around 30% of all cases of adult leukaemia, in Asian countries (and in Asian-American people) and Japan CLL is an infrequent disease and remains so in the migrant population.

Pathogenesis

Neoplastic CD5+ B CLL cells are antigen experienced and express CD23, CD25, CD27, CD69 CD71, ROR1 and CD200. It is likely that they originate in haematopoietic stem cells (HSCs) found amid clonal haematopoiesis. The evolution from these progenitors to MBL and, ultimately, to overt CLL is the result of additional genetic and epigenetic abnormalities, B-cell receptor (BCR) stimulation, and microenvironment and T-cells crosstalk. Relevant genetic drivers of the disease are the deletion of miR15 and miR16 in chromosome 13q14, trisomy 12 and *MYD88* mutations. Over time, other mutations appear, including those involving *TP53, ATM, BIRC3, NOTCH1, XPO1* and *SF3B1*. Finally, in the most advanced cases, *C-MYC* and *CDKN2A* mutations can also be present.

Importantly, the immunoglobulin heavy chain variable region genes (*IGHV*) may be mutated or unmutated. Mutated *IGHV* (*IGHV*-M) (defined as <98% homology to germline IGHV sequence) CLL originates from post-germinal centre (GC) $CD5^+CD27^+$ B cells. Conversely, *IGHV*-unmutated (*IGHV*-UM) CLL appears to derive from pre-GC $CD5^+CD27^-$ B cells, which might arise from naive B cells or a separate lineage of precursor B cells, in a T cell-independent process (Figure 23.1). Of note, the *IGHV* gene repertoire is enriched in specific subtypes (e.g. *IGHV1-69*, *IGHV3-7* and *IGHV4-34*), suggesting a role for antigen stimulation and selection in CLL pathogenesis.

A key feature in CLL pathogenesis is the progressive accumulation of CD5 + B cells because of a failure in programmed cell death or apoptosis. Although most cells are in $G_{0/1}$ phase, there is a fraction of cells (0.1–1%) rapidly duplicating and renovating every day. The resistance of these cells to apoptosis is related to the upregulation of the antiapoptotic proteins such as BCL2 and MCL1 and downregulation of proapoptotic proteins such as BCLX. The microenvironment plays an essential role in protecting CLL cells from death through several molecules. This is demonstrated by the fact that *in vitro* CLL cells rapidly undergo apoptosis unless they are co-cultivated with stromal cells or in a stromal-mimicking milieu.

Immune defects

Hypogammaglobulinaemia affects ~25% of patients and tends to worsen over the course of the disease. In early stage, the absolute count of $CD8^+$T lymphocytes is increased, causing an imbalance of the $CD4^+/CD8^+$ ratio. T cells show an abnormal phenotype, with increased expression of exhaustion markers such as cytotoxic T lymphocyte antigen-4, lymphocyte activation gene 3, CD244, CD160 and PD-1. Functionally, these phenotypic changes correlate with a deficient immune synapse formation, significantly reduced cytotoxic and proliferative potential. Defects in NK cells, ADCC, monocytes and granulocytes are also found. Response to vaccination is suboptimal.

Clinical features

Approximately, 80% of patients are diagnosed while asymptomatic. In the rest, painless generalised peripheral lymphadenopathy (i.e. neck, axillae, and inguinal) is frequent. In contrast, mediastinal and retroperitoneal lymphadenopathy are less common. On some occasions, the investigation of bacterial infections, particularly pneumonia, or herpes virus infections may lead to the diagnosis. Rarely, the disease is discovered during the diagnostic workup of an autoimmune haemolytic anaemia (AIHA) or, even less frequently, an immune thrombocytopenia (ITP). Fatigue, fever, weight loss and drenching night sweats may affect a significant proportion of patients. A peculiar feature in some patients is high sensitivity to insect, mainly mosquito, bites. Rarely, CLL may involve extra haematological tissues, such as skin, liver, kidney or central nervous system; in such cases, however, disease transformation needs to be ruled out. In addition, vasculitis, hypercalcaemia and nephrotic syndrome are occasionally observed. Spontaneous regression of the disease

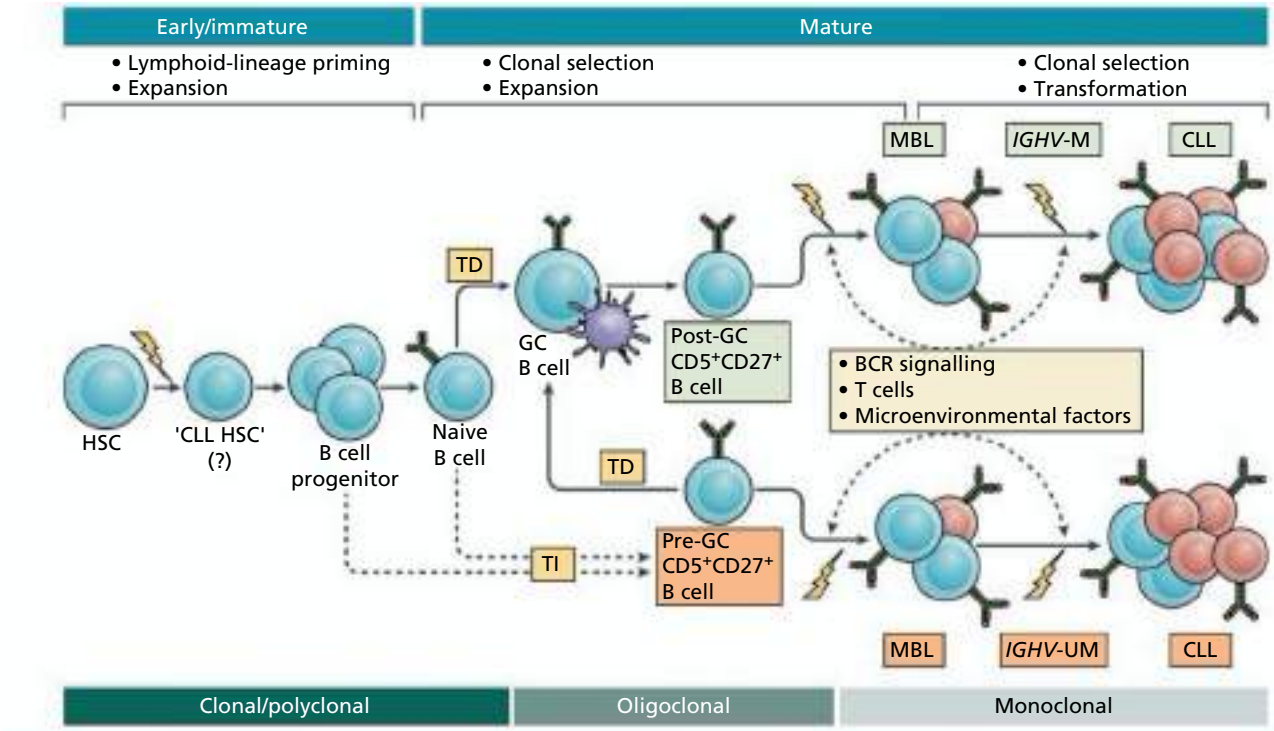


Figure 23.1 Genetic and epigenetic events leading to CLL. The genetic and epigenetic events leading to CLL have been suggested to occur in haematopoietic stem cells (HSCs). After HSCs acquire genetic and epigenetic lesions (indicated by lightning symbols), the cellular output of these lesions could be a polyclonal expansion of B cell progenitors. Subsequent antigenic stimulation might lead to oligoclonal selection and expansion of mature B cells. As mentioned above, CLL cells with mutations in the immunoglobulin heavy chain variable region genes (*IGHV*-M) seem to originate from post-germinal centre (GC) CD5+ CD27+ B cells, which are most likely derived from CD5+ CD27+ B cells that have undergone the GC reaction. Conversely, *IGHV*-unmutated (*IGHV*-UM) CLL seems to be derived from pre-GC CD5+ CD27− B cells, which might arise from naive B cells or from a separate lineage of precursor B cells, probably in a T cell-independent process. The evolution from these progenitors to monoclonal B lymphocytosis (MBL) and, ultimately, to overt CLL is dictated by additional genetic and epigenetic abnormalities (indicated by lightning symbols), B cell receptor (BCR) stimulation, microenvironmental factors and T cells. The dashed arrows indicate a hypothetical mechanism. TD, T cell-dependent antigen; TI, T cell-independent antigen (Source: From Bosch F and Dalla Favera R. Nature Reviews/Clinical Oncology 16 November 2019 (reproduced by permission)).

can be observed in 1–2% of patients per year. The remission, though, is rarely complete, most patients have persistent disease by flow cytometry and it is usually transient. The mechanisms behind spontaneous remissions are poorly understood but could involve immune-mediated reactions.

Patients with CLL may present concurrent diseases and fragility. The Cumulative Illness Rate Score (CIRS) is frequently employed to assess comorbidity. Particularly important is an impaired renal function (creatinine clearance <60 mL/min), which is associated with poor tolerance to fludarabine-based therapy and higher toxicity. Cardiac arrythmias, coagulation abnormalities and hypertension need to be taken into consideration when treatment with BTKi is considered.

Laboratory features

The hallmark of the disease is an increased white blood cell (WBC) count with a high percentage of lymphocytes and absolute lymphocytosis (Figure 23.2). A proportion of prolymphocytes (1–10%) are nearly always seen when WBCs are higher than 30×10^9/L. If the proportion of prolymphocytes is greater than 15% CLL is considered to be transforming into an aggressive (or accelerated) clinical form (Figure 23.3). Some patients have a mixed pattern of small and large cells and others have lymphoplasmacytoid features or even cells with nuclear clefts. These are often associated with other atypical features, such as $SmIg^{strong}$ and trisomy 12. *MYD88* L265P mutation is not detected.

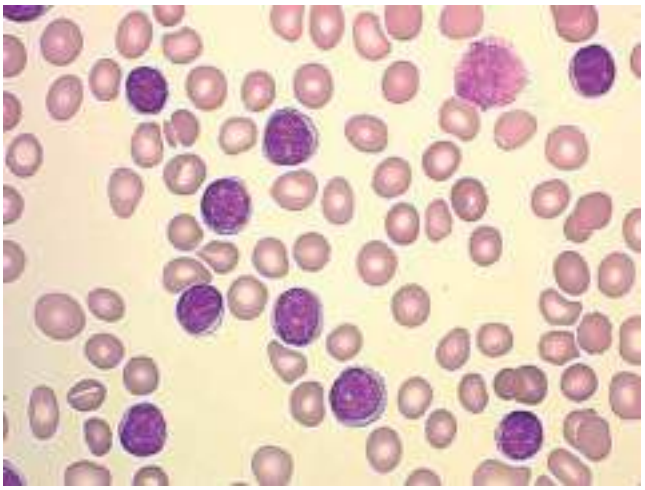


Figure 23.2 Blood film of a typical case of CLL. Small lymphocytes with scanty cytoplasm and tenuous nucleoli. In the upper right corner, a Gumprecht cell is observed.

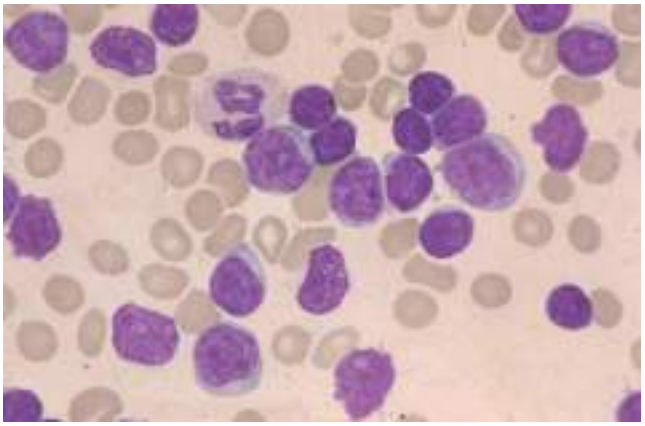


Figure 23.3 Blood film from a case of transforming CLL. Note the dual population of small lymphocytes and larger nucleolated prolymphocytes.

At diagnosis, anaemia is found in less than 10% of the subjects. Importantly, anaemia is not always due to the infiltration of the bone marrow by the disease; other causes such as autoimmunity, iron, folic acid or vitamin B12 deficiency may cause anaemia, particularly in the elderly and malnourished people. Likewise, thrombocytopenia is infrequent at diagnosis. A marked thrombocytopenia (e.g. $<20 \times 10^9$/L) in a patient with previous normal counts should raise the possibility of an immune origin, particularly in the absence of anaemia. Hypogammaglobulinaemia is frequent, particularly in advanced phases of the disease (20–40% of patients). IgM is the first immunoglobulin to decrease followed by IgG and, less frequently, IgA. Serum immunofixation can demonstrate an M component (usually of the IgM type) in 5–10% of cases. A positive direct antiglobulin test (DAT) is observed in around 5% of the patients at the time of diagnosis, with clinically apparent AIHA being less frequent.

Genetic and molecular features

CLL has no specific genetic lesions. The most frequent cytogenetic abnormalities are del(13q14), del(11q22-23), trisomy 12, del(6q) and del(17p13.1), which are associated with some peculiar clinical features and outcomes (Table 23.3). Del(17p) is observed in 5–50% of cases, depending on the characteristics of the studied population (e.g. untreated and treated). Historically, metaphase cytogenetic studies of CLL have yielded little information because CLL cells neither readily divide spontaneously in culture, nor do they respond to traditional B-cell mitogens. The stimulation of CLL cells with CpG oligodeoxynucleotide has facilitated the detection of cytogenetic abnormalities in up to 80% of patients. Thus, complex karyotypes (usually defined as either >3 or >5 aberrations) can be observed in 5–30% of patients, depending on the characteristics and phase of the disease, while chromosomal translocations are found in 20–30% of cases. In around 30% of cases, cytogenetic evolution occurs over the course of the disease. *TP53* mutations are usually observed in del(17p), but in 5–15% of cases, *TP53* mutations can occur in the absence of del(17p). Mutations of other genes such as *MYD88, NOTCH-1, SF3B1* and *BIRC3* can be observed in 5–15% of cases. Epigenetic lesions are the subject of many studies to better understand the landscape of genetic lesions in this disease. The *IGHV* gene repertoire is enriched by specific subtypes (e.g. *IGHV1-69*, *IGHV3-7* and *IGHV4-34*).

Pathology features

In typical cases, the histology of lymph nodes shows effacement of the architecture by small lymphocytes with a pseudo-follicular pattern of pale areas that correspond to proliferation centres. Proliferation centres contain numerous T cells, a fine network of dendritic cells and B cells with an increased expression of proliferation-associated markers and antiapoptotic molecules. There are patients whose lymph nodes have expanded proliferation centres (pseudo-follicles) with a high proliferation rate ('accelerated' or 'transforming' CLL). Differences observed in lymph node histopathology over the course of the disease are consistent with the concept of CLL as an evolving disorder, from typical CLL to 'accelerated'/'transforming' CLL and eventually disease transformation or Richter's syndrome (RS) (see Disease Transformation).

The spleen shows a diffuse infiltration, mainly affecting the white pulp.

The bone marrow displays a variable degree of infiltration by the disease but, in contrast to follicular lymphoma, there is no para trabecular infiltration. Four infiltration patterns have been described: nodular, interstitial, mixed (nodular + interstitial) and diffuse. While in early clinical phases of the disease nodular

Table 23.3 Cytogenetic and molecular features. Biologic and clinical correlations.

Abnormality	(%)	Correlations		Median survival (years)
		Biological	Clinical	
del(13q14) isolated	25–40	Mutated *IGHV* genes *MYD88* LD265P mutations Mir15, Mir16 expression	Good prognosis	>15
Trisomy 12	15–20	Unmutated *IGHV* genes Atypical morphology (prolymphocytes) Atypical immunophenotype (CD20+FMC7+, CD11c+) *NOTCH1* mutations	Short treatment-free survival after CIT Resistance to therapy, shorter survival	~10
del(11q22-23)	20–25	Unmutated *IGHV* genes *ATM* mutations (30%) *BIRC 3* mutations	Short treatment-free survival Longer PFS with FCR vs. FC or F BCRi/BCL2i overcome poor prognostic significance	6–8
del(17p13.1)	5–50	Unmutated *IGHV* genes *TP53* mutations Clonal evolution Complex karyotype	Short treatment-free survival Resistance to CIT BCRi/BCL2i overcome poor prognostic significance	3–6

BCL2i, BCL2 inhibitors; BCRi, BCR inhibitors; CIT, chemo(immuno)therapy; F, fludarabine; FC, fludarabine, cyclophosphamide; FCR, fludarabine, cyclophosphamide, rituximab; PFS, progression-free survival.

and interstitial patterns predominate, in advanced phase diffuse infiltration is the norm.

Diagnostic criteria

The diagnosis of CLL requires the following parameters:

- Presence in peripheral blood of $>5\times10^9$/L monoclonal B lymphocytes persisting for at least 3 months.
- Demonstration of the clonality of the population (κ/λ analysis).
- Characteristic immunophenotype: SmIg^{weak}, CD5$^+$, CD19$^+$, CD20^{weak} and CD23$^+$.

Based on the immunophenotypic characteristics and giving one point to each one of the following markers: CD5$^+$, CD23$^+$, FMC7^{weak}, SmIg (κ/λ staining) ^{weak} and CD79b^{weak}, Matutes and Catovsky showed that a score of 4–5 indicates almost invariably CLL, while a score <3 corresponds in most instances to non-CLL cases. FMC7 is a CD20 epitope and is now rarely employed as a marker. CD20 or CD79b can be used instead of FCM7 to obtain the diagnostic score. In line with this, the 'characteristic' CLL immunophenotype (i.e. SmIg^{weak}, CD5$^+$, CD19$^+$, CD20^{weak} and CD23$^+$) might be expanded to include other markers such as CD200.

Bone marrow aspirate and biopsy are not necessary for diagnosis but can be useful in complex cases and to provide information about the origin of cytopenias (i.e. bone marrow failure because of disease infiltration versus 'peripheral' mechanisms such as hypersplenism or autoimmunity). Moreover, the presence of cytopenia caused by a typical marrow infiltrate establishes the diagnosis of CLL regardless of the number of peripheral blood B lymphocytes or the lymph node involvement or organomegalies.

Differential diagnosis

Usually, the diagnosis of CLL is straightforward and does not present difficulties. In CLL, a small percentage (i.e. 1–10%) of atypical lymphocytes (e.g. prolymphocytes, lymphoplasmacytoid cells and cleaved cells or centrocytes) can be present in blood. If the percentage of atypical cells is >15% CLL is considered to be in 'transforming' or 'accelerated' phase. In such cases, trisomy12, FMC7 expression, *TP53* deletion, high rate of cell divisions (as shown with the antibody Ki-67) and many proliferating centres ('pseudo follicles') in lymph nodes and bone marrow can be found.

The diagnosis of SLL requires the presence of lymphadenopathy and the absence of cytopenias caused by a clonal marrow infiltrate. Additionally, the number of B lymphocytes in the peripheral blood should be $<5\times10^9$/L. In SLL, the diagnosis should be confirmed by histopathological evaluation of a lymph node biopsy or biopsy of other tissues. Some patients may present with enlarged lymph nodes that are not suspicious for solid tumours and with peripheral blood B lymphocytes $<5\times10^9$/L that carry a typical CLL immunophenotype. In these cases, a tissue or lymph node biopsy may not be necessary to establish the diagnosis of SLL.

Lymphoplasmacytic lymphoma (LPL) is characterised by a proliferation of *IGHV* mutated late-stage mature B-cells, with an admixture of small lymphocytes, plasmacytoid lymphocytes and plasma cells. A monoclonal IgM peak is a hallmark of Waldenström disease, a form of LPL. Neoplastic cells are IgM+, CD19+, CD20+, CD22+, CD25+, CD27+, FMC7+, CD5 variable, CD10−, CD23−, CD103− and CD108−. Plasma cells are typically CD138−. The diagnosis requires the exclusion of other B-cell disorders with plasmatic differentiation. *MYD*88(L265P) mutations are found in nearly all patients, and mutations of *CXCR4* are present in around 30% of cases. In some cases, a moderate increase of lymphocytes in blood is observed.

The diagnosis of *atypical CLL* should not be accepted without a comprehensive study to exclude a lymphoma in leukaemic phase. Because of this, when atypical lymphoid cells are present, it is important to consider the diagnosis of lymphoma evolving with tumour cells in blood. MCL can present with leukaemic involvement. The best method to exclude (or confirm) MCL is to test for t(11;14) by FISH and/or to demonstrate expression of cyclin D1 by immunohistochemistry. In contrast with nodal MCL, the leukaemic form is SOX (−) and CD200 (+). Other lymphomas can be excluded by histological criteria and the combination of peripheral blood morphology, immunophenotype and genetic features (Table 23.2).

A rare abnormality is *persistent polyclonal B-cell lymphocytosis*. This disorder is characterised by a median age at diagnosis of 40 years, a female predominance, a moderately increased and persistent lymphocyte count and occasional splenomegaly. The bone marrow is slightly infiltrated, sometimes showing intrasinusoidal distribution. Most patients are smokers and 90% have HLA-DR7 in their genotype. The cell markers show a non-clonal cell proliferation, $CD19^+$, $CD5^-$, $CD23^-$ and $CD27^+$. There are also increased levels of polyclonal IgM in serum. Despite its benign and non-clonal nature, several abnormalities have been shown in the B lymphocytes such as an extra copy of the long arm of chromosome 3 in the form of iso(3q) and also the presence of *IGH/BCL2* rearrangements. The blood picture is pleomorphic and frequently shows binucleated lymphocytes (Figure 23.4), which are characteristic of this condition. Persons with this abnormality should not receive therapy but be followed to detect an otherwise infrequent (around 3%) disease transformation.

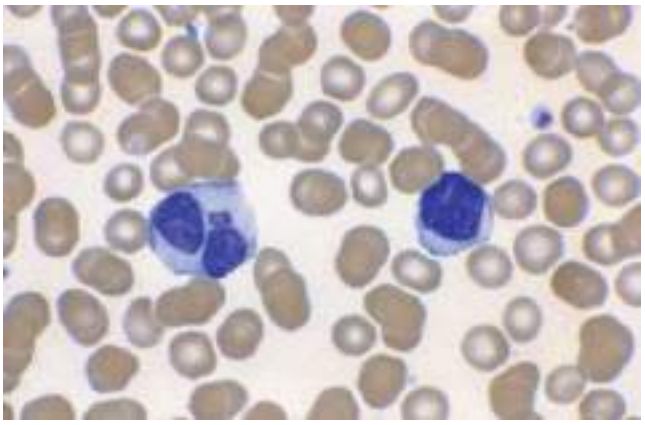


Figure 23.4 Blood film from a case of polyclonal B-cell lymphocytosis showing a binucleated cell characteristic of this condition.

Monoclonal B-cell lymphocytosis

In the absence of lymphadenopathy, organomegalies (detected clinically or by imaging studies), cytopenia and clinical symptoms, the presence of $<5\times10^9$/L monoclonal B lymphocytes in blood is defined as monoclonal B cell lymphocytosis (MBL). The prevalence of MBL in the normal population is 3–12% and is higher in first-degree relatives of patients with CLL and older subjects (13–18%). Its detection depends on the sensitivity of the flow-cytometry technique. The cut-off value of 5×10^9/L monoclonal cells for diagnosing MBL was selected arbitrarily to be consistent with CLL diagnostic guidelines and include patients in trials and is not based on substantial biological differences between MBL and Rai stage O CLL. Moreover, MBL represents a miscellaneous category ranging from patients with minute clonal B-cell populations detected in the general population or blood donors to patients referred for consultation. There are cases in which there is more than one B-cell clone, and others in which there is a mixture of monoclonal B cells and monoclonal T cells. Although the immunophenotype is in most cases identical to that of CLL, there are cases with an atypical immunophenotype (e.g. CD5-negative) or resembling SMZL, LPL, FL, non-nodal, leukaemic MCL or multiple myeloma, without fulfilling diagnostic criteria for overt malignancy.

MBL is classified by both the specific clonal B-cell immunophenotype (CLL-like MBL, non–CLL-like MBL or atypical MBL) and the size of the B-cell clone (low-count or high-count MBL). High-count MBL is defined by an absolute B-cell count $\geq0.5\times10^9$/L, makes up ~2% of all MBLs, and is typically identified in the clinic during the workup of lymphocytosis. High-count MBL has been shown to progress to CLL requiring

Table 23.4 Comparison of CLL, SLL and MBL.

	CLL	SLL	MBL
Clonal B-cells in peripheral blood	$\geq 5 \times 10^9$/L	<5x10^9/L	<5x10^9/L
Atypical cell immunophenotype	CLL/SLL	CLL/SLL	• CLL type • Non-CLL type • Atypical MBL
Anaemia and/or thrombocytopenia	Possible	Possible**	No
Lymphadenopathy*	Possible	Yes	No
Organomegaly*	Possible	Possible	No
Bone marrow involvement	Yes	Possible	Yes
Disease-related symptoms	Possible	Possible	No
Lymph node biopsy	Not required	Required	Not required

CLL, chronic lymphocytic leukaemia; SLL, small lymphocytic lymphoma.
* Imaging studies recommended.
** Cases with cytopenia caused by the infiltration of bone marrow by lymphocytes are categorised as CLL, independently of the presence or not of lymphadenopathy and/or organomegaly.

treatment at a rate of 1–2% per year. Conversely, the clonal population can spontaneously regress over time. From the clinical standpoint, MBL has been associated with a higher tendency to suffer infections, immune defects and second neoplasias, although no striking differences exist with what occurs in Rai stage 0 CLL. Persons with MBL should be reassured their condition only needs periodic monitoring and that the possibility of evolving to CLL is remote. Terms such as 'leukaemia', 'pre-leukaemia' or 'cancer' should not be unnecessarily used when explaining the nature of the disease to the patient.

In Table 23.4, the differences between CLL, SLL and MBL are shown.

Autoimmune cytopenia

CLL is frequently accompanied by autoimmune cytopenia. *Autoimmune haemolytic anaemia* (*AIHA*) occurs in around 10% and immune thrombocytopenia (ITP) in 5–7% of the patients; *pure red-cell aplasia* (*PRCA*) and immune neutropenia are rare (<1% of the patients). AIHA can appear before or concurrently with the diagnosis of CLL, spontaneously over the course of the disease, while the patient is on therapy, or thereafter. AIHA and ITP may present together (Evan's syndrome).

The diagnosis of *AIHA* should be suspected in presence of anaemia of abrupt presentation or a significant worsening of previous anaemia accompanied by elevated LDH with or without increased bilirubin, low or undetectable haptoglobins and positive DAT. The examination of a peripheral blood smear is mandatory since it usually shows spherocytes. The absolute reticulocyte count may not be striking due to poor bone marrow function because of leukaemic infiltration or treatment toxicity. DAT is usually positive for IgG/IgG+C3. Cold-agglutinin disease (CAD) has also been described in CLL patients, in which case DAT will only show positivity for C3d, with agglutination at low temperature and in a few cases weak positivity for IgG.

It is accepted that treatment can trigger the appearance of AIHA, a fact already mentioned in seminal descriptions of CLL. However, the 1990s notion that fludarabine frequently caused AIHA was probably overrated as it was based on retrospective studies in highly selected, and heavily pre-treated patients. In fact, randomised studies in untreated patients demonstrated that the proportion of patients treated with fludarabine + cyclophosphamide (FC) or FC + rituximab (FCR) developing AIHA is significantly lower (1–5%) than with chlorambucil alone (12%) or fludarabine alone (11%). Targeted therapies (i.e. BCRi and BCL2i) are associated with AIHA in 2–5% of cases.

Immune thrombocytopenia (ITP) is most commonly an incidental finding on a routine blood count. Diagnosing ITP may present difficulties because there is no sensitive and specific diagnostic test. Nevertheless, thrombocytopenia can be considered immune-mediated when platelet counts suddenly decrease in the absence of splenomegaly, infection or CIT and the bone marrow shows abundant megakaryocytes. Staining with Factor VIII may help to reveal the presence of megakaryocytes in a massively infiltrated marrow. In advanced CLL, anaemia usually occurs before thrombocytopenia. Therefore, isolated thrombocytopenia, particularly if extremely low, is

more likely to be immune in origin. Response to corticosteroids may be the final, *post-hoc* diagnostic test. In those areas in which this is a prevalent problem, *Helicobacter pylori* infection should be excluded. Treatment should be that of primary ITP.

Erythroblastopenia or PRCA is defined by the lack, or maturation arrest, of red blood cell precursors in bone marrow, anaemia and low absolute reticulocyte count. In the presence of anaemia, the reticulocyte percentage can be misleadingly normal; therefore, the reticulocyte percentage corrected according to the haematocrit or, even better, the absolute reticulocyte count is more informative. The bone marrow shows characteristic defects of erythroblast maturation. In the presence of a bone marrow heavily infiltrated by lymphocytes, the identification of red cell precursors can be difficult. In this setting, anti-glycophorin immunohistochemistry may facilitate the identification of red cell precursors. Importantly, PRCA can be seen in association with other immune cytopenias, particularly AIHA. In addition, any patient with CLL and anaemia with a low reticulocyte count should be evaluated for viral infections that can be associated with PRCA, namely *cytomegalovirus, Epstein–Barr virus* and *parvovirus*.

Autoimmune neutropenia is exceedingly rare (<1%). Likely, most early reports of this complication did not occur in the context of *bona fide* CLL cases, but of misdiagnosed T-cell large granular lymphocytosis.

Prognosis

The *prognostic significance* of AIHA is controversial, but in some studies that when patients with advanced clinical stage (Binet C, Rai III, IV) are stratified according to the mechanism of the anaemia, those with 'immune' advanced stage do better than those with 'infiltrative' advanced stage, in which the cytopenia is due to a packed bone marrow. The reason for this difference, of important consequences in prognosis and clinical management, is patients with autoimmune cytopenia tend to respond well to corticosteroids, while those with a high tumour burden do not always respond satisfactorily to CLL therapy.

Treatment

Treatment of patients with CLL and autoimmune cytopenia is largely based on experts' opinions and analysis of retrospective series. In those patients with AIHA in the context of inactive CLL, the treatment should be the same as in idiopathic AIHA: initially with corticosteroids, and then, in patients who fail to respond or relapse quickly, anti-CD20 monoclonal antibodies. If there is no response to these two approaches, alternative immunosuppression (e.g. cyclosporine and azathioprine) should be considered. Good results have also been achieved with rituximab plus corticosteroids; rituximab, cyclophosphamide and dexamethasone; or rituximab, dexamethasone and cyclosporine.

For patients with ITP, corticosteroids are the mainstay of therapy with a response rate of around 80% after 1–3 weeks of therapy. Dexamethasone yields responses more quickly than prednisone but the long-term results are not different. Once a response is achieved the dose of corticosteroids should be tapered over a period of 4–8 weeks. Around 30% of patients are refractory to corticosteroids or suffer early (<6 months) relapse. Rituximab, cyclosporine and mycophenolate can rescue patients unresponsive to corticosteroids. The thrombopoietin receptor agonist eltrombopag may be effective in ITP associated with CLL, as in primary ITP, and is increasingly used as second-line therapy. Splenectomy can be considered in individual cases impossible to control by other measures. Intravenous immunoglobulin is of help when a rapid response is needed (e.g. a patient with thrombocytopenia and significant mucosa bleeding, pre-surgery), though as a single agent, it does not produce lasting effects. Antiplatelet agents such as aspirin, clopidogrel and prasugrel should not be given (or should be discontinued).

Supportive care should include blood product transfusion as clinically indicated, folic acid in AIHA and local measures to control bleeding in ITP.

Importantly, failure of autoimmune cytopenia to respond to conventional treatment is an indication for anti-CLL therapy.

Non-haematological autoimmunity is exceedingly rare, the only disorders that have been reliably linked to CLL being cold-agglutinin disease, paraneoplastic pemphigus and acquired angioedema.

Second neoplasias

Around 10–15% of patients with CLL develop other cancers. Melanoma, lung carcinoma, lymphoma, Kaposi sarcoma, CNS and gastrointestinal tumours are the cancer types most frequently observed. Skin cancers other than melanoma can be also found but at a frequency no different from that of the general population. Patients with CLL have a substantially increased risk for a rare skin tumour known as Merkel cell carcinoma, and *vice versa*. It is usually considered that there is no relationship between treatment and the incidence and type of secondary solid tumours. However, patients heavily pre-treated with cytotoxic agents (e.g. FCR) may present with secondary MDS/AML (.5% of cases).

Infections

Infections are frequent and the commonest cause of death in patients with CLL (40–60%). Their pathogenesis is multifactorial, including low IgG levels, impaired antigen presentation, immunosuppression and treatment-related myelotoxicity. Most infections are bacterial and frequently involve the respiratory tract. IgG replacement may be useful in patients with profound hypogammaglobulinaemia and repeated bacterial infections.

The pathogenesis of infections with purine analogues is related to T-cell abnormalities induced by these agents, with herpes virus infections being frequent. Infections by *Pneumocystis jirovecii, Listeria, Mycobacteria, Aspergillus* and *Candida* can be also observed. Importantly, anti-CD20 monoclonal antibodies (e.g. rituximab and obinutuzumab) carry a risk of hepatitis B reactivation amongst patients positive for hepatitis B surface antigen (HBsAg) or antibodies against hepatitis B core antigen (anti-HBc). All patients should be screened for hepatitis B before starting treatment. Patients with evidence of prior hepatitis B infection should be monitored for clinical and laboratory signs of reactivation during therapy and for several months after its completion. Anti-CD20 monoclonal antibodies should be discontinued in patients with hepatitis re-activation. Finally, although the overall risk of contracting COVID-19 does not appear to be different from the general population, patients with CLL have a higher risk of suffering severe forms of the disease.

Vaccination with inactivated high-dose influenza vaccines, haemophilus influenza, herpes zoster and conjugate pneumococcal vaccine may reduce the risk of infections. However, the serological response to such vaccines is low, particularly in previously treated patients and those treated with anti-CD20 monoclonal antibodies within the first year of exposure. Likewise, SARS-Cov-2 vaccination show a suboptimal response, but this is not a reason to omit vaccination; on the contrary, double vaccination is recommended.

Disease transformation (Richter's syndrome)

CLL can transform into B-cell diffuse large cell lymphoma (DLCL), an event historically known as Richter's syndrome (RS). RS occurs in about 5% of patients. There is no relationship between treatment modality and the risk of RS, but some studies suggest a higher risk of RS in treated patients. *IGHV* genes are frequently unmutated. Other biomarkers associated with RS are *NOTCH1*, *TP53, CDKN2A/B, MYC* mutations and stereotyped *IGHV* genes (#8 configuration). Akt activation may behave as an initiator of CLL transformation by inducing Notch signalling between RS cells and T cells.

RS is heterogeneous in its biology and prognosis. In 80% of cases, the transformation occurs in the CLL clone. The prognosis of these patients is poor, with a median survival inferior to 1 year. In the remaining 20% of patients, the DLCL arises in a CLL-unrelated B-cell clone, and the prognosis of these patients is like those with *de novo* DLCL.

Disease transformation should be suspected whenever the patient suffers a sudden worsening of the general status, fever, enlarging lymph nodes or extra nodal involvement. A sharply increasing LDH level is an important clue to suspect disease transformation. RS is often associated with monoclonal protein in the serum or free light chains in the urine. RS can be reliably suspected on clinical grounds but needs to be confirmed by biopsy. The diagnosis, however, may be difficult because RS can be localised in an isolated organ (e.g. spleen, lymph nodes of a given territory or in extra lymphatic tissues). Rarely, the transformation takes place in the bone marrow; in such cases, the large blast-like cells spill over to the peripheral blood and the morphology may resemble that of an acute leukaemia (Figure 23.5). 'Accelerated' CLL, in which a high proportion of proliferation centres ('pseudo follicles') are observed in lymph nodes should not be confused with RS, its prognosis being intermediate to classic CLL and RS. In contrast to uncomplicated CLL, PET/CT can show areas of hyperactivity corresponding to the transformed tissue, where it is more likely to identify RS.

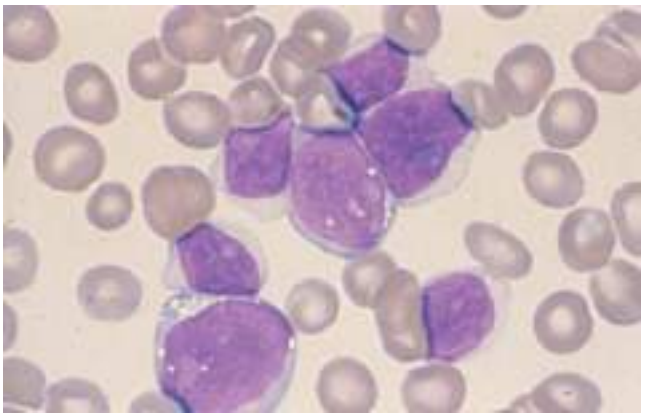


Figure 23.5 Large, blast-like atypical cells corresponding to a case of Richter syndrome with blood involvement.

Besides DLCL, cases of transformation into Hodgkin's lymphoma can be observed (5–10% of all RS cases). These are mainly observed in older men presenting with mixed-cellularity histological subtype and can be EBV-driven.

The treatment strategies for RS vary according to the type of disease transformation. In those cases in which the emerging DLCL is CLL-clonally related, the prognosis is poor (survival <1 year) and allogeneic stem cell transplantation (allo-HSCT) should be considered. Treatment with new BCR and BCL2 inhibitors and CAR T-cells is being investigated. In contrast, CLL-clonally unrelated DLCL has good prognosis and should be treated as *de novo* DLCL (e.g. R-CHOP). The prognosis of transformation into Hodgkin's lymphoma is heterogeneous and treatment should be risk-adapted; there are cases responding well to standard Hodgkin's lymphoma regimens.

Prognosis

The median survival of patients with CLL has improved over the last years and is now >10 years. This is mainly due to better therapies and an earlier diagnosis of the disease in routine blood analysis. The individual prognosis, however, is heterogeneous and ranges from a few months to a normal lifespan. This,

coupled with the lack of a truly curative therapy, makes prognostication an essential component in the management of patients with CLL.

Although somewhat overlapping, it is useful to distinguish parameters that predict disease progression, and hence need for therapy (*prognostic factors*), and those informing about the probability that an individual patient will respond to a given therapy (*predictive factors)* (see Treatment).

Importantly, the mere correlation of a parameter with a given outcome does not qualify it as an outcome marker. Outcome markers need to fulfil a number of requisites to be considered as such. These include standardisation, inter- and intra-patient reproducibility, specificity and sensitivity, identification of a substantial proportion of patients, independent prognostic value from other biomarkers for the same event and usefulness to manage patients. Moreover, determining outcome markers is only justified if patient's management will depend on their results.

Progression-free-survival (PFS) (a most frequent endpoint in clinical trials) is a treatment-related variable (the more effective the treatment, the longer the PFS) and, consequently, does not necessarily correlate with overall survival (OS), which depends on the effect of different treatments given during the course of the disease. Likewise, response predictors vary in function of the treatment modality.

Clinical stages

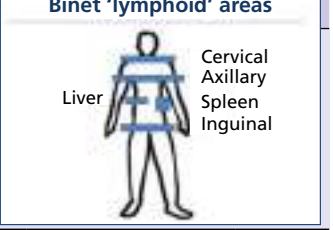
The clinical stages independently formulated by Rai and Binet in the 1970s continue being useful as a raw estimate of the tumour bulk. Clinical stages are based on the concept that neoplastic CLL cells accumulate first in blood, subsequently in lymph nodes and spleen, and eventually in bone marrow, leading to its functional failure (Table 23.5). In historical series, patients with low-risk disease (Binet A, Rai 0,) have a median survival longer than 15 years, those with intermediate-risk disease (Binet B, Rai I, II) have a median survival of 6–8 years and patients with high-risk disease (Binet C, Rai III, IV) have a median life expectancy of less than 5–6 years. Assigning a clinical stage to a given patient only requires a physical examination and a complete blood cell count; such simplicity is a great advantage that facilitates the application of clinical stages worldwide. Of note, Binet stages can be subclassified according to Rai stages, thus refining their prognostic significance (i.e. better survival: A0>AI>AII; BI>BII). The prognostic significance of clinical stages is steadily vanishing because of effective therapies.

Cytogenetics

Most patients with CLL (~90%) present with cytogenetic abnormalities that can be detected by FISH. Trisomy 12 and deletions at 13q14.2-q14.3 (including *DLEU2*, *MIR15A*/*MIR16-1*), 11q22.3 (*ATM*) and 17p13.1 (*TP53*) are the most frequent abnormalities. All these aberrations have important clinical correlates (Table 23.3). Döhner *et al*., in a seminal publication, proposed a hierarchical model of cytogenetic lesions for prognostic purposes. This model implies that patients are classified according to the worst cytogenetic abnormality, as shown in Table 23.3, independently of the number of lesions. It should be noted that del (13q14), present in up to 50% of cases, confer a good prognosis, provided they are found as the only change. Del(11q22-3) frequently involves the *ATM* gene and its prognostic significance mainly depends on whether or not the gene in the other allele is mutated. Del(17p3.1) is found in 5–30% of cases, its prevalence being higher in patients with advanced and heavily

Table 23.5 Rai and Binet clinical stages.

Rai	**0**	**I**	**II**	**III**	**IV**
Blood lymphocytosis	Yes	Yes	Yes	Yes	Yes
Lymphadenopathy	No	Yes	Yes/No	Yes/No	Yes/No
Spleen/liver enlargement	No	No	Yes	Yes/No	Yes/No
Hb <110 g/L	No	No	No	Yes	Yes/No
Platelets $<100 \times 10^9$/L	No	No	No	No	Yes
Median survival (years)	>15	10–11	7–9	3–5	3–5
Binet	**A**	**B**	**C**	**Binet 'lymphoid' areas**	
Blood lymphocytosis	Yes	Yes	Yes		
<3 lymphoid areas* enlarged	Yes	No	Yes/No		
> 3 lymphoid areas enlarged	No	Yes	Yes/No		
Hb <110g/L and/or Platelets $<100 \times 10^9$/L	No	No	Yes		
Median survival (years)	>15	7–9	3–5		



* cervical, axillary, inguinal lymph nodes (either uni- or bilateral), spleen and liver.

pre-treated CLL. In the case of complex karyotypes (defined as >3 or >5 genetic aberrations), the lesions usually include *TP53* mutations. Del (11q) and particularly *TP53* mutations are a strong predictor of response to CIT, while their impact on patients treated with BCRi or BCL2i is much lower (see Response biomarkers).

IGHV gene mutational status

The *IGHV* gene can be either mutated (<98% homology to the nearest germline sequence) or unmutated in a proportion of 50–60% and 30–50%, respectively. Importantly, *IGHV* mutational status separates CLL into two different clinic-biological forms. While patients with mutated *IGHV* genes (*IGHV*-M) usually have indolent disease not needing therapy and good prognosis, those with unmutated *IGHV* genes (*IGHV*-UM) tend to have rapidly aggressive disease, respond poorly to therapy and have short survival. *IGHV* mutational status correlates with other outcome biomarkers (Table 23.6). *IGHV* mutational status is thus not only a major marker of two different forms of disease, but also a central prognostic feature around, which revolve many other prognostic factors. The *IGHV* gene repertoire is enriched by specific subtypes (e.g. *IGHV1-69*, *IGHV3-7* and *IGHV4-34*). Some of these subgroups share a similar prognosis. For example, *IGHV3-21* gene usage (of stereotype subset # 2) may be associated with an unfavourable prognosis independent of the *IGHV* mutational status. As of today, assessment of *IGHV* stereotypes is not an element of the routine prognostic work up in CLL. Notably, the prognostic significance of *IGHV* mutational status was identified in cohorts of patients treated with CIT and has been virtually overcome with the use of BTKi and BCL2i, as discussed later (Table 23.6).

Gene mutations

The del(17p) at the *TP53* locus is observed in 5–50% of cases, depending on the clinical situation (i.e. early, untreated disease versus late, heavily treated and resistance to CIT) and in most cases correlates with a point mutation in the other allele, resulting in total inactivation of the *TP53* suppressor gene. In 2–5% of patients, however, *TP53* mutation can be present in the absence of del(17p). Prognostically, *TP53* mutations are the most important predictor of response to CIT. *TP53* mutations can be observed in small CLL subclones, bearing the same poor prognosis (see Response predictors). In turn, del(11q) deletions are usually monoallelic, frequently large and include a minimally deleted region (MDR) which encompasses the *ATM* gene in 30–40% of cases. *BIRC3*, a negative regulator of non-canonical NFKB signalling located at 11q22, has been found to be deleted or mutated in a mutually exclusive manner with *TP53*. *NOTCH1* and other mutations have been correlated with outcome and response to therapy. BTKi mutations and *BCL2* mutations are associated with resistance to therapy with ibrutinib and venetoclax, respectively. Not surprisingly, the higher the number of mutations and distorted genetic pathways, the poorer the prognosis. The importance of gene mutations in response to therapy is further discussed in the Treatment section.

Prognostic systems and scores

Prognosis is related to many factors, and thus a single parameter rarely predicts an outcome. Because of this, the prognostic assessment is based on the combination of different parameters in the form of stages, prognostic groups or scores.

Table 23.6 *IGHV* gene mutational status: biological and clinical correlates.

Mutated *IGHV*	Unmutated *IGHV*
Early stage (A, O)	Advanced stage (C, III, IV)
WBC count $<30\times10^9$/L	WBC count $\geq30\times10^9$/L
LDT >12 months	LDT ≤12 months
Low-risk genetics	High-risk genetics
del(13q)(isolated)	del(17p)
MYD88 mutations	del(11q)
CHD2 mutations	(+12)
Normal karyotype	Complex karyotype
	TP53, NOTCH1, SF3B1, BIRC3, POT1, XPO1 mutations
Low ZAP-70	High ZAP-70
Low B2M	High B2M
Low CD38	High CD38
Low thymidine-kinase	High thymidine-kinase
Median survival >15 years	Median survival: 6–7years

The CLL-International Prognostic Index (CLL-IPI) includes Rai stage, age, *TP53* status, *IGHV* mutational status and serum B2M and distinguishes four different prognostic subgroups predicting time to first therapy and OS. However, this and other similar models were developed in patients treated with CIT and poorly predict the outcome of patients receiving targeted agents such as BCRi or BCL2i. For example, age, Rai stage and *IGHV* mutational status have *no* prognostic significance in patients treated with ibrutinib, in whom adverse biomarkers are del(17p)/*TP53* mutation, high B2M, prior therapy and time interval from last therapy <24 months.

Moreover, prognostic and predictive models or scores apply to groups of patients but not to individual persons, and are far from being perfectly accurate. Thus, the most widely used accuracy indicator is the C-index in which a value of 0.0 indicates no accuracy at all, 1.0 is tantamount to a perfect prediction and 0.5 is like flipping a coin. The C-index of most outcome models is 0.70–0.80, meaning that the prediction may be wrong in 20–30% of the patients. These caveats emphasise that outcome markers complement, but cannot replace, clinical expertise and a thorough clinical judgement.

In Table 23.7, the most important outcome predictors for disease progression in global series of patients with CLL are shown.

As most patients with CLL are diagnosed in asymptomatic, early phases of their disease (Binet A, Rai 0) several models to predict disease progression in such patients have been proposed to (i) inform patients, (ii) decide frequency, modality and setting for patients' monitoring and (iii) select patients for clinical trials. Table 23.8 shows parameters associated with disease progression in asymptomatic Binet stage A disease according to different studies; such markers are otherwise superimposable to those in general series of patients.

Table 23.7 Prognostic biomarkers.

Age
Clinical stages
***IGHV* mutational status**
Del(17p)/*TP53* mutation
Del (11q)/*ATM* mutation
Complex karyotype
LDT
B2M
ZAP70
LDH
Thymidine-kinase
PFS or time from last therapy <24 months

B2M, Beta 2 microglobulin; LDT, lymphocyte doubling time; PFS: Progression-free-survival.

Table 23.8 Biomarkers associated with disease progression in asymptomatic Binet A/Rai 0 disease.

Unmutated *IGHV*
Del(17p)/*TP53* mutation
Del(11q)/*ATM* mutation
B2M
LDT
>60 years old
Nodal involvement (Rai substage*)
Increased blood lymphocyte count (i.e. >15 x10^9)

B2M, Beta 2 microglobulin; LDT, lymphocyte doubling time; PFS, Progression-free-survival.
* AO versus AI versus AII; BI vs BII.

Prognostic assessment in daily practice

In daily practice, the large and ever-increasing number of reputed outcome biomarkers can end up being, quite understandably, more confusing than helpful. Because of this, physicians routinely seeing patients with CLL should be aware that early clinical stage (Binet A, Rai 0), mutated *IGHV* genes, good-risk cytogenetics [del(13q)], normal serum B2M, low blood lymphocyte count (e.g. $<15\times10^9$/L) and a long LDT (e.g. >12 months) indicate good prognosis, with a survival close to the general population. And that advanced clinical stage (Binet B/C, Rai I–IV), unmutated *IGHV* genes, poor cytogenetics [del(11q), del(17p), complex karyotype], increased B2M, high blood lymphocyte count (e.g. $\geq15\times10^9$/L) and short LDT (e.g. <12 months) are associated with poor prognosis; of note, all these parameters are easily available in almost all locations. Likewise, physicians should keep in mind that currently the only biomarkers with a direct influence on treatment selection are unmutated *IGHV* and poor genetic features [i.e. del(17p)/*TP53* mutations; del(11q)]. Patients with any of these abnormalities should be treated with pathway inhibitors (i.e. BCRi or BCL2i) and not CIT.

Treatment

Criteria to start therapy

- Treatment should be only initiated in case of active/progressive disease (PD) as defined by the following parameters:
- Anaemia (Hb<110 g/L) and/or thrombocytopenia (platelets $<100\times109$/L) due to marrow failure.
- Massive (i.e. ≥6 cm below the left costal margin) or progressive or symptomatic splenomegaly.
- Massive (i.e. ≥10 cm in longest diameter) or progressive or symptomatic lymphadenopathy.

- Progressive lymphocytosis with an increase of ≥50% over a 2-month period, or lymphocyte doubling time (LDT) of <6 months.
- Causes of reactive lymphocytosis (e.g. infections and steroid administration) should be excluded. LDT is rarely (<10% of cases) the only criterion to start therapy.
- Autoimmune complications including anaemia or thrombocytopenia poorly responsive to standard treatment (i.e. corticosteroids).
- Symptomatic extra haematopoietic involvement (e.g. skin, kidney, lung or spine).
- Disease-related symptoms as defined by any of the following:
  - Unintentional weight loss ≥10% within the previous 6 months.
  - Significant fatigue (i.e. Eastern Cooperative Oncology Group performance status (ECOG-PS) ≥2: cannot work or unable to perform usual activities).
  - Fever ≥38.0 °C for ≥2 weeks without evidence of infection.
  - Night sweats for ≥1 month without evidence of infection.

Since the diagnosis of CLL rarely constitutes a medical emergency, the need for therapy and its modality should be decided after 1–3 months of observation, a period in which the diagnostic workup can be completed, the pace of the disease evaluated and the goals of therapy discussed with the patient.

Younger age or adverse biomarkers are not per se criteria to initiate treatment. Likewise, in the absence of other features of active disease, a high WBC count or hypogammaglobulinaemia with no infections do not justify treatment. In CLL, leukostasis due to a high WBC is exceedingly rare. However, if the WBC is $>300 \times 10^9$/L, a short intervention with chlorambucil or oral fludarabine to decrease the WBC and comfort the patient may be justified. As supported by several studies, patients benefit from being managed by experienced doctors at CLL referral centres.

Pre-treatment evaluation

The pre-treatment evaluation should include a complete medical history, physical examination to detect enlarged lymphoid areas, complete blood cell counts, DAT, renal function, general biochemistry and immunoglobulin levels. CT scans are not part of the routine assessment and should only be performed if clinically indicated (e.g. suspected retroperitoneal, lung or mediastinal involvement). A bone marrow biopsy can provide important information about tumour burden and origin of cytopenias, and is useful as baseline information. A normal creatinine clearance (>60 mL/min.) is required if fludarabine-based therapy is to be given, and hepatitis B serology should be performed in patients in whom treatment will contain an anti-CD20 antibody. Assessing comorbidity and frailty (geriatric assessment) is important since most patients with CLL are older.

Predictive biomarkers

The OS of CLL patients requiring treatment depends on the response to therapy and its degree. Patients achieving CR with undetectable minimal residual disease (uMRD or 'negative' MRD) upon therapy have an excellent outcome. Achieving uMRD CR largely overcomes the impact of adverse biomarkers. Predictive factors indicate the probability of response to a specific therapy. Importantly, genetic abnormalities, which are the most important predictive factor, can change over time in approximately 30–40% of patients; because of this, their study should be performed before initiating therapy, and should be repeated before changing treatment during the course of the disease. The most important predictive biomarker is del(17p)/*TP53* mutation that predicts a low probability of achieving a good and sustained response with CIT (i.e. chlorambucil; chlorambucil + rituximab; fludarabine; FC; FC + rituximab). This scenario has changed; thanks to the introduction of BTK inhibitors (BTKi) (i.e. ibrutinib and acalabrutinib), PI3K-delta inhibitors (i.e. idelalisib) and BC2L inhibitors (BCL2i) (i.e. venetoclax) that abrogate the negative impact of adverse response biomarkers, although the results are somewhat inferior in del(17p)/*TP53*-mutated cases. Likewise, unmutated *IGHV* predicts a shorter PFS in patients treated with CIT but not in those receiving small molecules. *NOTCH1*, *SF3B1* and *BIRC3* mutations have been correlated with inferior response to CIT, but they are not routinely tested. Patients carrying *NOTCH1* mutations do not benefit from adding anti-CD20 monoclonal antibodies to FC but this marker is not employed in daily practice. In patients treated with BTKi (i.e. ibrutinib and acalabrutinib) or BCL2i (venetoclax), *BTK* and *BCL2* mutations can be the cause of resistance to therapy and, hence, are worth of being determined in cases of treatment refractoriness. Of note, such mutations can be detected much time before actual clinical refractoriness appears. Besides abnormalities detected by FISH analysis, a complex karyotype (as defined by >3 or >5 abnormalities) is a biomarker of poor response to therapy and short OS (Table 23.9).

Minimal residual disease

Achieving a state of uMRD upon therapy is clinically important because patients in CR with uMRD have a longer PFS and OS than those with persistent MRD, independently of the treatment given. However, MRD is an arbitrary concept that depends on the technique used to assess it. Besides, high sensitivity flow-cytometry (FCM), high throughput sequencing can be useful to refine MRD detection. There are several factors such as type of therapy (containing or not anti-CD20 antibodies), site where MRD is evaluated (blood or bone marrow), time point at which MRD is assessed that complicate cross-comparisons of different studies. Pursuing MRD eradication as the treatment endpoint and MRD-guided therapies are only justified within clinical trials.

Table 23.9 Biomarkers predicting response to therapy. Differences between chemo(immuno)therapy and pathway inhibitors.

Biomarker	Chemo(immuno) therapy	Pathway inhibitors
Del(17p)/*TP53* mutation	+++	+/−
Del/11q)/*ATM* mutation	++	−
IGHV mutational status	++	−
Complex karyotype	++	+/−
B2M	+	+/−
LDT	−	−
NOTCH1 mutations	+	NA
BTK mutations	Not applicable	++
BCL2 mutations	Not applicable	++

B2M, Beta 2 microglobulin; LDT, lymphocyte doubling time; PFS, Progression-free survival.

Objectives of treatment

CLL largely remains an incurable disease. The objectives of therapy are therefore to prolong survival (which ultimately depends on the response to different therapies administered over the course of the disease) and improve quality of life. Of note, endpoints used in clinical trials (e.g. CR rate, uMRD, PFS and time to next treatment [TTNT]) are not equivalent to treatment objectives, although there is a strong correlation between uMRD CR and OS.

Treatment approaches

Representative results that have contributed to build-up current treatment strategies in CLL are shown in Table 23.10.

As treatment modalities for CLL are ever-changing, patients should be included in clinical trials whenever possible. For patients not included in trials, the main treatment strategies are discussed below.

Management of asymptomatic disease

Large proportion of patients with CLL (40–60%) are asymptomatic at diagnosis. Several studies have shown that treatment of asymptomatic disease with agents such as chlorambucil, fludarabine or FC+rituximab results in a longer PFS but not a longer OS. The standard management of asymptomatic disease is, therefore, active surveillance. Several prognostic models based on *IGHV* mutational status and FISH cytogenetics [(del(17p)/del(11q)], may help to identify patients with early stage (Binet A, Rai 0) and different time-to-first therapy (TTFT) and OS. (see Prognosis and Table 23.8). These models are useful to inform patients, plan the frequency of follow-up visits (usually every 3–6 months initially and every 12 months thereafter) and select subjects for clinical trials. Monitoring should consist of physical examination and the assessment of blood cell counts, basic biochemistry, LDH serum levels and LDT. Imaging studies (e.g. CT) have no role in routine visits and should only be performed if clinically indicated.

Management of symptomatic disease

Front-line treatment

Chemo(immuno)therapy

The standard intervention has been CIT, namely the combination of fludarabine, cyclophosphamide and rituximab (**FCR**). The response rate with FCR is over 90%, including 30–40% of patients achieving uMRD. In a long-term follow-up of 300 patients initially treated with FCR, the 6-year OS was 77%, the median time to progression was 80 months and the treatment-related deaths <1%. Moreover, 50–60% of the patients with mutated *IGHV* and no adverse genetic features may remain in remission for >10 years ('functional' cure). However, FCR can only be safely given to younger persons (i.e. <65 years), fit, with no important comorbidity and normal renal function (creatinine clearance >40 mg/L). This excludes the majority of patients with CLL because they are older than 70 years, present comorbidities and have poor general status.

Frequent toxic events include fever and infections (including opportunistic), which may be observed up to 2 years after finishing treatment. There are other complications, such as HBV reactivation and, rarely, progressive multifocal leukoencephalopathy (PML), linked to treatment with rituximab. MDS/AML can be observed in a proportion (around 5%) of heavily pre-treated patients.

Beside FCR, other CIT regimens have been developed aiming at maintaining efficacy and reducing toxicity. The combination of **bendamustine and rituximab (BR)** is a popular regimen to treat patients >65 years old, although it is not as effective as BTKi (see below).

Although defining who is an older person is difficult and subject to frequent revision, 65–70 years of age is the cut-off used to separate 'younger' from 'older' patients. Although chronological age is less important than 'biological' age, only a small proportion of subjects older than 65–70 years can safely receive intensive cytotoxic therapy because of frailty or comorbidity. A Cumulative Index Rating Score (CIRS) >6 is used in some studies to identify patients for whom intensive treatment is not advised. With the increasing life expectancy of the general population, the group of very old patients

Table 23.10 Representative clinical trials in CLL.

	Patient number	Patients' treatment Status (TN or R/R)	Median Follow-up	ORR (CR) (%)	PFS (median/%)	OS (median/%)
FCR	408	TN	5.9 years	90 (44)	56.8 months	82% (@ 5 years)
BR	279	TN	58.2 months	96 (31)	42.3 months	80.1% (@ 5 years)
Chlorambucil + Obinutuzumab	238	TN	30 months	55 (22.3)	26.7 months	NA
Ibrutinib	195	R/R	74 months	83 (2)	44.1 months	67.7 months
Ibrutinib (2 trials)	136	TN	82.7 months	86–93 (4–7)	59% (at 7 years)	78% (at 7 years)
Ibrutinib + Rituximab	182	TN	55 months	94 (12)	76% (at 4 years)	86% (at 4 years)
Ibrutinib + Obinutuzumab	113	TN	45 months	91 (42)	74% (at 42 months)	88% (at 2 years)
Ibrutinib + Rituximab	352	TN	5.8 years		78% (at 5 years)	95% (at 5 years)
Acalabrutinib	155	R/R	46.5 months	86 (1)	62% (at 42 months)	78% (at 42 months)
Acalabrutinib	179	TN	74.5 months	82	62% (at 72 months)	76% (at 72 months)
Acalabrutinib + Obinutuzumab	179	TN	74.5 months	945 (13)	78% (at 30 months)	84% (at 72 months)
Idelalisib + Rituximab	110	R/R	18 months	85.5	Median 20.8 months	Median 40.6 months
Venetoclax	127	R/R	14 months	79 (20)	25 months	84% (at 24 months)
Venetoclax + Rituximab	194	R/R	59.2 months	92.3 (26.8)	Median 53.6 months	82% (at 5 years)
Venetoclax + Obinutuzumab	216	TN	76.4 months	88	76.2 months	78.7% (at 6 years)
Zanubrutinib	214	TN No (17p+)	18 months	94.5	88.6% (at 24 months)	95.5% (at 24 months)

Note: This table is not intended to be a comprehensive analysis nor a cross-comparison of different trials, its purpose is merely informative.
Regular updates of CLL treatment results may be found at National Comprehensive Cancer Network (NCCN) (www.nccn.org/guidelines); European Society of Medical Oncology (ESMO)
www.esmo.org/guidelines; and National Institute for Health and Care Excellence (NICE) (www. nice.org.uk.guidance).

(e.g. >80 years) is increasing. This group calls for a cautious, tailored management based on a geriatric assessment and with quality of life as treatment goal; the inclusion of these patients in clinical trials is only justified if the treatment investigated is relatively non-toxic. Historically, chlorambucil has been the drug given to patients who cannot safely receive more intensive treatments. The dose usually given ranges from 40 to 70 mg/m^2 per month and is given intermittently (i.e. 10 mg/m^2 per day for 7 days or 0.5 mg per kg every 2 weeks, in monthly courses); the duration of the treatment depends on the degree of response but usually ranges from 6 to 12 months. Chlorambucil in association with anti-CD20 antibodies yields better results than Chlorambucil alone. Chlorambucil yields a small proportion of CRs (5–10%), and although it improves symptoms, survival is not substantially prolonged. However, in line with what has been discussed above, chlorambucil can be a plausible treatment in very old patients (e.g. >80 years) in which the aim of therapy is symptom control.

Targeted therapies

The advent of targeted agents has revolutionised treatment algorithms for CLL by allowing the use of chemo-free, generally well tolerated, and effective regimens for a wider range of patients than CIT candidates, and with better results. Importantly, targeted agents may overcome the negative impact of unmutated *IGHV* and del(17p)/*TP53* mutations on outcome.

Ibrutinib is a selective irreversible (covalent) BTK inhibitor, which is given continuously at a dose of 420 mg/day until disease progression or unmanageable toxicity. Ibrutinib given as single agent results in a 70–90% ORR in the front-line setting and 40–70% in previously treated patients. The CR rate increases over time (up to 35% in previously untreated subjects). Long-lasting responses are observed in patients without previous treatment (5-year PFS ~75%), whereas patients with relapsed or refractory CLL have shorter remissions (~40%). Importantly, ibrutinib may abrogate the poor prognostic impact of biomarkers such as unmutated *IGVH* and del(11q) and, to a lesser

degree, del(17p)/*TP53* mutations, particularly in previously treated patients. A series of randomised clinical trials have shown better outcomes in patients receiving ibrutinib alone or in combination with CD20 monoclonal antibodies as compared to chlorambucil ± anti-CD20 monoclonal antibodies, BR and FCR, mainly in patients with unmutated *IGHV*. The main toxicities of ibrutinib include infections, hypertension, atrial fibrillation and bleeding. Ibrutinib has shown to be effective in patients relapsing after venetoclax (and *vice versa* venetoclax is effective in patients refractory to ibrutinib). Altogether, around 50% had to discontinue ibrutinib therapy because of toxicity or disease progression. The time interval between treatment discontinuation and disease progression has been reported to be 25 months. Resistance to ibrutinib is usually linked to BTK^{C481S} mutation and *PLCG2* mutations, which occur in around 30% of patients.

Acalabrutinib is another irreversible BTK inhibitor, which shows a higher selectivity for BTK than ibrutinib. In clinical trials, acalabrutinib monotherapy or in combination with obinutuzumab significantly improved PFS as compared to obinutuzumab plus chlorambucil in elderly patients; and compared to rituximab with either idelalisibib or BR. Frequent side effects include hypertension, bleeding, atrial fibrillation, diarrhoea, headache and respiratory tract infection, although to a lesser degree than ibrutinib due to reduced off-target kinase inhibition. Therefore, acalabrutinib is a good alternative to ibrutinib when this latter agent causes unmanageable toxicity (but not in case of refractoriness). Results of trials comparing ibrutinib with acalabrutinib suggest similar efficacy but a lower frequency of cardiac events with acalabrutinib.

Zanubrutinib is another non-covalent BTKi recently approved by FDA and EMA for CLL treatment. As compared to ibrutinib and acalabrutinib treatment results are similar, if not better, and adverse events (i.e. hypertension, cardiac events), less frequent.

Pirtobrutinib is a new generation, non-covalent, BTKi which has demonstrated promising results in patients with relapsed/refractory disease, with an ORR of around 60% and a PFS of around 70% at 2 years from treatment initiation, including in patients previously treated with covalent BTKi showing BTK^{C481S} and *PLCG2* mutations Ongoing randomised studies will determine the position of pirtobrutinib in CLL treatment algorithms.

Idelalisib is an oral reversible inhibitor of phosphoinositide 3-kinases (PI3Ks), which inhibits the signalling of the BCR and other surface receptors. Because of inflammatory immune-mediated complications (hepatitis, colitis and pneumonitis), especially in untreated CLL patients and infections (i.e. *P. jirovecii* pneumonia and CMV reactivation), and lower efficacy when compared to BTKi, idelalisib is not used as front-line therapy but can be an alternative in patients who cannot tolerate BTKi. Treatment discontinuation has been reported in up to 80% of the patients, in most instances (~50%) because of toxicity.

Venetoclax is an orally administered, highly selective potent BCL-2 inhibitor, targeting the anti-apoptotic BCL-2 protein, which is present in high amounts in CLL cells. It yields a high CR rate (40–80%), including a substantial proportion of uMRD. To prevent tumour-lysis syndrome, venetoclax is initially administered following a weekly dose ramp-up schedule. Frequent severe adverse events include neutropenia, infections, anaemia and thrombocytopenia. Venetoclax can be used with obinutuzumab in patients who have not previously been treated or with rituximab in patients who have received at least one previous treatment. It can also be used on its own in·patients with del(17p)/*TP53* mutation) who cannot be treated with or are refractory to BCRi (ibrutinib and idelalisib); and patients who do not have these genetic changes, after treatments with chemoimmunotherapy as well as BCRi have both not worked. In clinical trials, the combination of venetoclax with ibrutinib and obinutuzumab has been shown to be extremely active in ongoing trials. Likewise, the 'triplet' venetoclax, ibrutinib and obinutuzumab has recently been shown to be highly effective in patients <70 years and no del(17) (uMRD CR:92.2%; PFS at 3 years 90.5%). Given these results, venetoclax-based combinations are likely to become the treatment of choice in many treatment settings, including upfront.

The main limitation of treatment with venetoclax is disease progression (~25% of cases). Resistance to therapy is usually associated with the acquisition of the Gly101Val point mutation in *BCL2* or other *BCL2* mutations at clonal and subclonal levels. However, these mutations are not found in all patients resistant to venetoclax, and other mechanisms, such as the upregulation of antiapoptotic proteins MCL-1 and BCL-xL might cause treatment resistance. In such cases, ibrutinib can be effective.

Front-line therapies for patients not included in trials are shown in Figure 23.6.

Relapsed/refractory disease

Importantly, disease relapse is not by itself a criterion to re-initiate treatment, only patients with relapsed *and* PD needing therapy (see Criteria to start therapy). There are patients with relapsed disease but no signs of progression in whom therapy can be postponed for prolonged periods of time. *TP53* status, serum B2M, advanced clinical stage, Hb level, LDH and time since last therapy <24 months are the most consistent outcome biomarkers in relapsed/refractory patients.

The introduction of pathway inhibitors (BCRi and BCL-2i) in CLL therapy has dramatically changed treatment strategies for this form of leukaemia, both upfront, as previously discussed, and at disease relapse. In patients having received effective

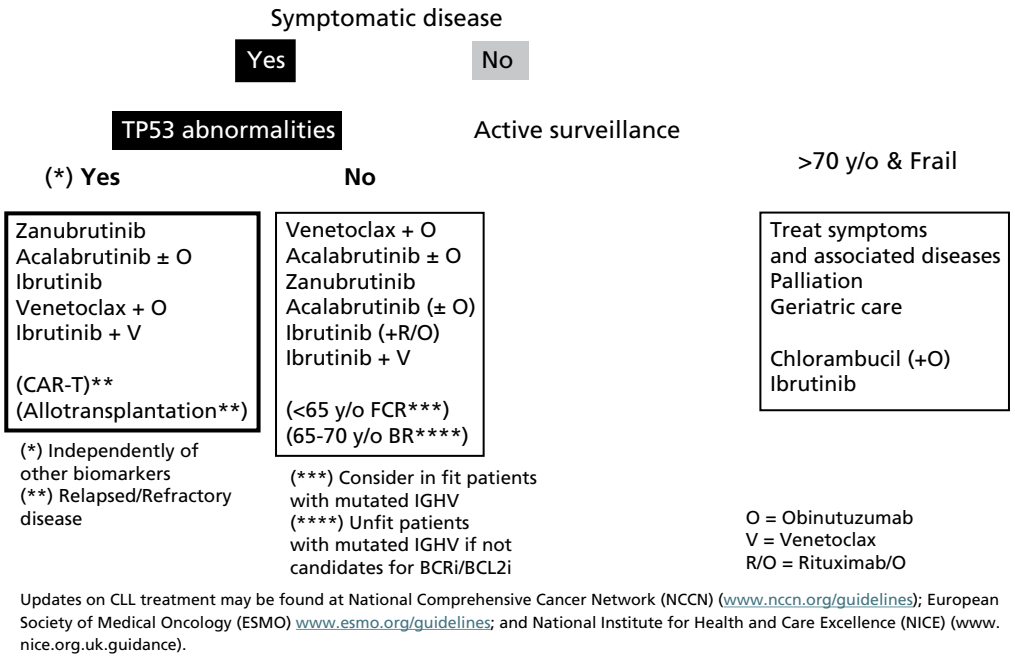


Figure 23.6 Treatment for patients with CLL.

front-line therapy (e.g. FCR), a PFS inferior to 2–3 years indicates poor-risk disease. Conversely, in patients with a longer PFS, the prognosis is considered to be better. In these latter patients, repeating the initial therapy has been considered a reasonable treatment approach. This notion, however, was generated when treatment possibilities for CLL were limited and poorly effective. Although repeating the initial therapy may be an option in selected patients, changing the type of therapy is in most cases a more logical approach.

Haematopoietic stem cell transplantation

While autologous transplants are considered not useful and have been virtually abandoned, allo-HSCT still has a role in the management of selected patients with CLL.

Allo-HSCT should be considered as an option in younger, fit patients with high-risk diseases having failed one or two pathway inhibitors and having a low transplant risk. According to the available evidence, fully myeloablative regimens offer no advantage over reduced-intensity conditioning regimens. Allo-HSCT exerts its therapeutic effect through the T-cell anti-leukaemia-mediated effect (graft-versus-leukaemia) and overcomes the negative impact of poor prognostic factors such as del(17p)/*TP53* mutations. The procedure-related mortality is around 10% at 1 year and 30% at 3 years from the transplant. Most deaths are due to graft-versus-host-disease and its complications. Disease relapses can be observed but are rare beyond 4 years from transplant. Transplant results are better in younger patients, with no comorbidity, transplanted in remission and receiving a transplant from a fully matched HLA donor.

Prior therapy with BTKi or BCL2i does not jeopardise transplant results. On the contrary, ibrutinib has been shown to be a safe and effective therapy as a 'bridge' to allo-HSCT. The experience with BCL2i is more limited and inconclusive. For idelalisib, an EBMT study has shown that prior therapy with this agent seems to increase the risk of suffering severe GVHD. Allo-HSCT should not be unnecessarily delayed nor considered as the last, desperate attempt to control the disease in refractory and heavily pre-treated patients, when the possibility of a successful transplant is remote.

CAR-T cells

T cells expressing CARs are highly targeted, move to tumour sites, undergo *in vivo* expansion and persist for long periods of time (Chapter 6). The most frequent antigen target for CAR-T cells is CD19. CART cells can trigger cytokine-release syndrome (hypotension, rigours, muscle aching, fever and anorexia) and CNS toxicity. To enable CAR T-cell expansion, chemotherapy is given before the infusion of CAR-T cells. Other important toxic events are profound and long-lasting hypogammaglobulinaemia and B-cell depletion. In CLL, despite overall response rates (ORR) of 60–95%, including MRD clearance in a large proportion of patients, the durable CR rates are relatively low, and only few long-term responses have been reported,

probably because of CLL-related immune defects that render CAR-T cells poorly effective.

Further studies refining CAR-T cell therapy for CLL are needed. Also, clinical expertise and a broad range of supporting measures are needed to carry out this form of therapy. CAR-T cells should only be given in the context of clinical trials in patients in whom allo-HSCT is not feasible or conveys a high risk to the patient.

Response categories

The following response categories are recognised:

- Complete response (CR)
  - CR undetectable ('negative') MRD
  - CR detectable ('positive') MRD
- Complete response incomplete (CRi)
- Partial response (PR)
- Stable disease (SD)
- Progressive disease (PD)

CR requires normalisation of blood counts and normal bone marrow as assessed by biopsy. Since clearing bone marrow disease can take time, the bone marrow biopsy should be performed 3 months after completing therapy. In clinical trials, it is recommended to further assess CR by measuring MRD. There are two types of CR: MRD-negative (undetectable) and MRD-positive (detectable), the outcome of patients with the latter not being as good as in those with uMRD CR. CT scan should be repeated only if abnormal at the start of therapy. Incomplete CR denotes cases with persistent anaemia, thrombocytopenia or neutropenia, due to myelotoxicity, who otherwise fulfil the criteria for CR. If the bone marrow is hypocellular, it is recommended the biopsy be repeated once the blood counts have recovered. Not infrequently, the degree of response improves over time. PR requires the regression of at least 50% organomegalies and abnormal blood cell counts. SD is defined as no major changes in the signs and symptoms present before starting therapy. PD is the appearance of signs or symptoms of active disease not present before starting therapy. Of note, treatment with BCRi results in a transient blood lymphocytosis that should not be confused with treatment failure and disease progression.

Other B-cell chronic disorders with leukaemic expression

Splenic marginal zone lymphoma

Two-thirds of patients with splenic marginal zone lymphoma (SMZL) (around 2% of all lymphomas) have circulating villous lymphocytes. However, a minority do not have villous cells and in some, the diagnosis can only be established after splenectomy.

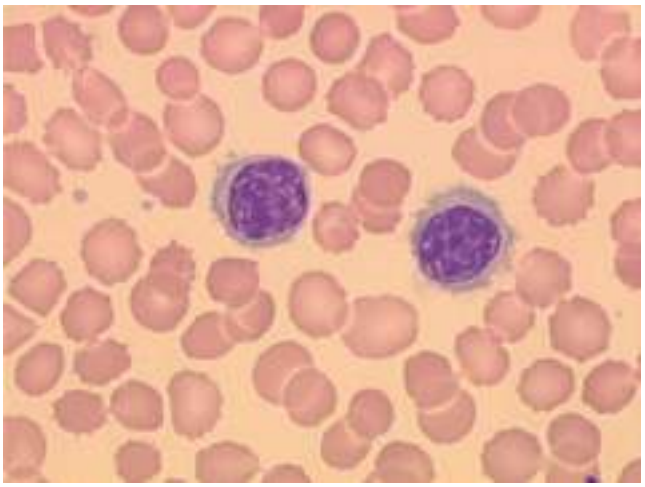


Figure 23.7 Lymphocytes from a case of SMZL. Villous lymphocytes are observed.

The median age of patients is around 70 years and the majority present with splenomegaly. Anaemia and/or thrombocytopenia are seen in 40% of cases. One-third have a serum monoclonal band, usually IgM below 30 g/L. The diagnosis is suspected on a typical blood film (Figure 23.7) showing small lymphocytes with an irregular membrane outline and short villi, often confined to one pole of the cells. The immunophenotype (see Table 23.2) is different from CLL. SMZL cells are negative for CD103 and CD123 but are always positive for CD11c.

Bone marrow aspirates may show the same cells as in the blood, with variable degrees of infiltration. A distinct pattern of intrasinusoidal infiltration is characteristic of SMZL, which can be highlighted with anti-B-cell antibodies (CD20 and CD79a). This pattern of infiltration is less common in other lymphomas and is not seen in CLL. The bone marrow infiltration can be nodular, interstitial and/or para trabecular. The spleen histology shows a characteristic bizonal pattern in the white pulp, with a central zone of small lymphocytes with scanty cytoplasm and a peripheral zone of larger cells with more dispersed chromatin and more abundant cytoplasm. The red pulp is always infiltrated by both the smaller and the larger cells. Plasmacytoid differentiation may be seen and transformation into large-cell lymphoma occurs in 10% of cases.

There is no unique cytogenetic abnormality in SMZL, but unbalanced translocations and deletions of 7q22–32 have been described in about 30% of cases. About 50% have a monoallelic deletion at 13q14. Trisomy 3 is found in around 15% of cases. Abnormalities of *TP53* are present in around 15% of patients and are associated with worse prognosis. The *MYD88* (L265P) mutation is found in almost 100% of cases of lymphoplasmacytoid lymphoma but is uncommon in SMZL.

An association with hepatitis C has been reported, particularly in the form of mixed cryoglobulinaemia, but its incidence

is unknown. Interferon (IFN)-α has been reported to induce CR in some hepatitis-C-positive SMZL patients. More recently, it was demonstrated that eradicating hepatitis C with direct active antivirals induced lymphoma responses in a proportion of patients. An association of SMZL with hyperreactive malaria and tropical splenomegaly has been described in African cases.

Rituximab is highly effective to reduce the spleen size and to control the disease and has virtually replaced splenectomy as mainstay of therapy. The latter, however, can be useful in cases in which there is not a good response to rituximab and can be a manoeuvre useful to establish the diagnosis and control the disease. In most cases, the OS is at least 10–15 years (see Chapter 27).

Hairy-cell leukaemia

Definition

HCL is an uncommon clinicopathological entity characterised by the accumulation in peripheral blood, bone marrow and splenic red pulp of small mature B lymphoid cells with abundant cytoplasm and 'hairy' projections. This, along with a reduction in normal blood cells, causes splenomegaly, anaemia, bleeding and an increased risk of infection. The so-called atypical or variant form of HCL (HCLv) was considered a provisional new entity in the 4th edition (2017) of the WHO classification system but in the 5th edition (2022) has been encompassed within a new entity: 'splenic B-cell lymphoma/leukaemia with prominent nucleoli'. Irrespective of the nomenclature, it should be separated from classical HCL (HCLc).

Epidemiology

HCL accounts for 2% of all leukaemia and affects males more frequently than females (ratio 4:1), usually over the age of 50 years. In a French study comprising the period 1996–2016, HCL represented 0.7% of all malignant haematological disorders and 3.0% of all leukaemias. The overall age-standardised incidence ratio (SIR) was 0.39/100,000 inhabitants in men and 0.09/100,000 in women, and it remained stable over the 20-year period analysed.

Pathogenesis

The *BRAF* V600E mutation is present in virtually 100% of cases of classical HCL and activates the RAF-MEK-ERK pathway leading to cell proliferation and accumulation. Also, important in the pathogenesis of HCL are several microenvironment-mediated cytokines and the exhaustion of the microenvironment itself.

Clinical features

In many cases, the diagnosis is made following investigation of a moderate pancytopenia. Fatigue is frequent and often underestimated. Other presenting features include infection (including opportunistic), bruising or mucosal bleeding. Splenomegaly is a characteristic feature but because of the now earlier diagnosis of the disease may be small or clinically undetectable, as compared to historical series (one-third of patients versus two-thirds). Lymphadenopathy is rare at diagnosis but can be observed in advanced and relapsed disease. Extranodal infiltration and nephrotic syndrome have been reported in a few cases. In contrast to CLL, the incidence of second cancers in patients with HCL does not appear to be increased compared to that of the general population.

Laboratory features

Anaemia is due to reduced bone marrow production and splenic pooling; haemolysis is exceptional. The WBC count may be low, normal or high (rarely $>20 \times 10^9$/L), but neutropenia and monocytopenia are almost always present, as is an increased MCV. Platelet counts are below 100×10^9/L in most cases.

The recognition of typical hairy cells in peripheral blood films is useful for suggesting the diagnosis. However, since the number of hairy cells in blood is typically low, a high degree of suspicion is required to make the diagnosis. Hairy cells are large, twice the size of a normal lymphocyte, with a barely discernible nucleolus and abundant cytoplasm that is characteristically villous ('hairy') in its outline (Figure 23.8). The nucleus is round, oval or slightly indented, and occasionally bilobed.

The typical HCL immunophenotype includes pan-B cell markers (CD19, CD20 and CD22) with co-expression of CD11c,

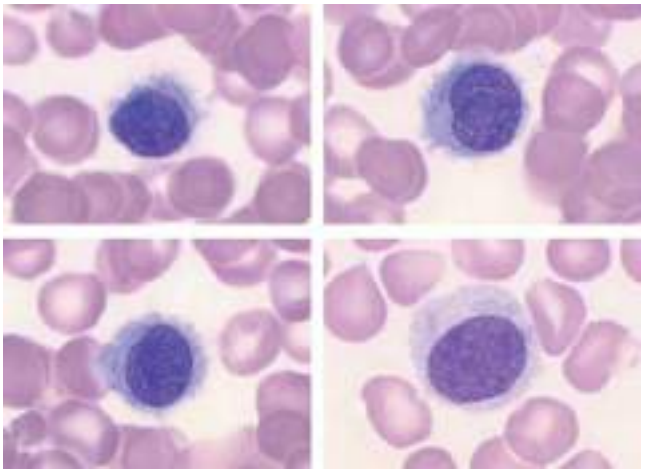


Figure 23.8 Individual hairy cells from blood films from two patients with HCL.

CD25, CD103, CD123 and CD200. *BRAF* mutations are found in virtually all patients (Table 23.2). Most cases (>80%) display mutated *IGHV* genes.

In most cases, bone marrow aspirates are unsuccessful ('dry tap') or yield scarce cellularity. The bone marrow biopsy shows diffuse interstitial infiltration of variable degrees; occasionally the infiltration is focal. A characteristic feature is the arrangement of the cellular infiltrate, which is loose, leaving plenty of space between cells, often with a clear zone around each cell, which is characteristic of this condition (Figures 23.9 and 23.10). Reticulin is always increased. Hairy cells stain strongly with anti-CD20. In 10–20% of cases, the bone marrow can be markedly hypocellular, which along with the pancytopenia may raise the possibility of aplastic anaemia as a diagnosis. In cases with hypoplastic marrows, immunohistochemistry (e.g. CD20, Annexin D1, DBA44, CD11c and VE1) is important for demonstrating clusters of hairy cells. Immunohistochemistry is also important to study MRD.

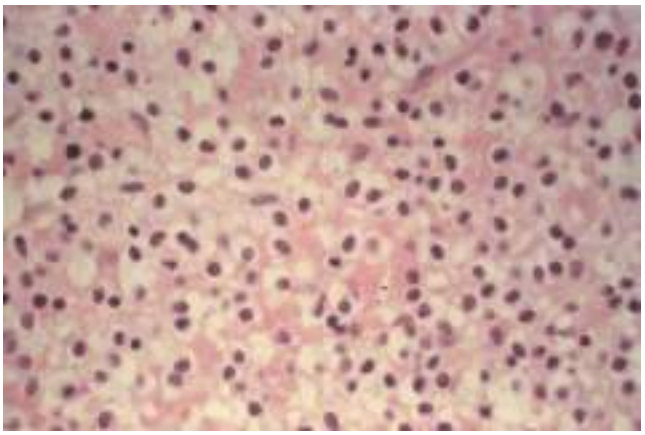


Figure 23.9 Bone marrow trephine section of a case of HCL showing the typical clear zone around the hairy cells in a paraffin-embedded section.

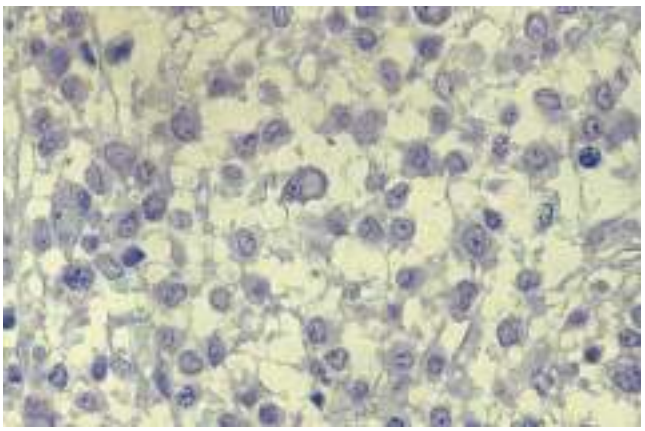


Figure 23.10 Bone marrow trephine from a case of HCL: section embedded in methacrylate giving good morphological detail of the hairy cells.

The spleen histology shows infiltration by mononuclear cells with a blunt nucleus in the red pulp with little residual white pulp, and formation of pseudo sinuses filled with erythrocytes.

The differential diagnosis of HCL in its classical form (HCLc) includes HCL atypical or HCLv, LPL, SMZL, B- cell prolymphocytic leukaemia (B-PLL) and splenic diffuse red pulp small B cell lymphoma (SDRPL) (Table 23.2).

Treatment

Treatment is only indicated in presence of active disease, as indicated by any of the following:

- Haemoglobin ≤110 g/L
- Platelet count $<100 \times 10^9$/L
- Absolute neutrophil count $<10 \times 10^9$/L.
- Splenomegaly
- Constitutional symptoms (i.e. fever, night sweats, fatigue and weight loss)

However, around 10% of patients present moderate asymptomatic pancytopenia and may be followed with no need for therapy for many years.

Front-line therapy

The purine nucleoside analogues, cladribine and pentostatin (PT), are the standard front-line treatment, with an ORR of 90–100% [complete response (CR) 70–90%] and no significant difference between the two agents. Remissions are durable (median, 15 years), and survival is close to that for an age-matched general population. In patients presenting with infection, this should be treated before commencing therapy. IFN (which is less myelotoxic than purine analogues) may be an option in case therapy cannot be postponed.

Cladribine (2-CDA) is effective in different schedules and by different routes (e.g. intravenous continuous infusion for 7 days, intravenous infusion over 2 hours on a 5-day regimen or alternatively subcutaneously on a once-per-day or once-per-week regimen.

PT is administered as a short 2 hours IV infusion every 2–3 weeks until remission is achieved (usually 8–10 injections). The dose may need to be modified based on renal function. Patients can experience nausea for up to 72 hours after the infusion.

Both pentostatin and cladribine cause temporary myelosuppression and a more prolonged immunosuppression. GCSF and prophylactic anti-infective agents (i.e. cotrimoxazole and acyclovir) may be initiated during treatment and continued for up to 6 months, or until adequate neutrophil and lymphocyte recovery. In the case of 2-CDA, it has been recommended to be

started after the 5-day course of treatment, since rashes can occur when the drugs are given concurrently. Allopurinol is not required.

Regarding classical therapies, splenectomy is now rarely performed because of the effectiveness of purine analogues in reducing the size of the spleen. Likewise, IFN is currently rarely given because of its side effects and inferior effectiveness in comparison with purine analogues. However, IFN may occasionally be useful in patients with serious infections and severe pancytopenia.

Rituximab monotherapy has not been formally tested in the first-line setting, but, based on the relatively low response rates seen in relapsed HCL, is not recommended as an alternative to purine analogues. Its use is reserved for patients unable to tolerate purine analogues or who present with an active infection. Nevertheless, rituximab may improve the remission rate when added to other agents. A study comparing 2-CDA with either concurrent or delayed rituximab (up to ≥6 months after detection of MRD in blood) resulted in a higher undetectable MRD CR rate in the concurrent arm. The use of this combination front-line is now standard in some centres, whereas others reserve the use of rituximab combinations for relapsed disease.

Treatment of refractory/relapsed disease

Resistance to therapy is exceedingly rare and should raise the possibility of an erroneous diagnosis. Patients with relapsed disease should only be treated if presenting symptoms, as in previously treated patients. Since almost all cases have a *BRAF* mutation, *BRAF* inhibitors (i.e. vemurafenib ± rituximab and dabrafenib) are effective to treat patients not responding to purine analogues or in relapse. Ibrutinib has been shown to be effective in this setting.

Non-nodal, leukaemic mantle cell lymphoma

Mantle cell lymphoma (MCL) signifies 3–10% of all lymphomas. MCL mainly affects older adults (median age, 60–65 years) with a male predominance. Broadly, two clinical forms of MCL are recognised: (i) nodal MCL (see Chapter 20) and non-nodal, leukaemic MCL included as an entity in the 5th WHO Classification 2022. Nevertheless, some degree of blood involvement can be demonstrated in almost 100% of cases if sensitive enough flow cytometry and molecular techniques are used. Close to 40% of cases evolving with splenomegaly have significant lymphocytosis, sometimes mimicking CLL (Figure 23.11). Most tumours have a classic morphology of small–medium-sized cells with irregular nuclei. However, the malignant lymphocytes may present with a spectrum of morphological variants, including small round (resembling CLL),

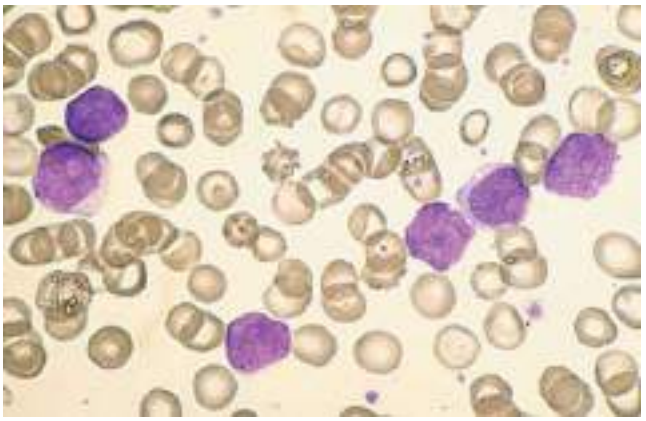


Figure 23.11 Blood film from a patient with mantle-cell lymphoma cells in peripheral blood. The cells are medium size, slightly irregular in shape and have a speckled nuclear chromatin pattern.

marginal zone-like, pleomorphic and blastoid cells. Blastoid cells, a high mitotic index and Ki-67 positive cells are all poor prognostic biomarkers. MCL are CD201, CD51 and positive for Cyclin D1 while being negative for CD10 and Bcl6. The hallmark chromosomal translocation t (11:14) (q13; 32) leads to the aberrant expression of cyclin D1 and identifies nodal. A few cyclin D1 negative cases have been identified. Overexpression of the transcription factor SOX11 has been described as a diagnostic marker for nodal MCL but is absent in non-nodal, leukaemic MCL. Biologic features such as the number of mitotic cells, a high Ki-67 proliferation index, *TP53* mutations and p16 deletions (*CDKN2A* mutation) are frequent in aggressive MCL subtypes such as the blastoid variant. On the contrary, low-density lipoprotein receptor protein 1 (LRPAP1) autoantibodies, present in around 13% of patients, correlate with a better outcome.

Marker studies show that MCL cells are always positive for CD5, CD19 and CD20, but negative for CD23 and CD200, although the latter may be absent in the leukaemic form (Table 23.2). The bone marrow biopsy shows a rather monotonous infiltration by slightly irregular cells. In contrast with CLL, no proliferating centres are seen. The pattern of bone marrow infiltration is often diffuse in advanced cases, but in other instances is nodular and/or paratrabecular (see Chapter 20).

The natural history of MCL often includes the worst features of both aggressive and indolent lymphoma: a rather aggressive clinical course and a pattern of resistant and relapsing disease with no sustained remissions. Historically, the median survival was 4–5 years; however, this is now 8–12 years; thanks to the availability of better therapies. On the other hand, not all cases of MCL require therapy at diagnosis. There are clinical forms biologically characterised by the expression of CD200, mutated *IGHV* genes, no del(17p)/*TP53* mutations and no expression of

SOX-1 in which the clinical course can be relatively indolent; these cases can be followed with no therapy for months, and their outcome is superimposable to those treated immediately after diagnosis. Treatment is as in nodal MCL.

Follicular lymphoma

Around 15% of patients with follicular lymphoma may have circulating lymphoma cells ($5–20 \times 10^9$/L). The proportion of patients with inconspicuous blood involvement is much higher, as demonstrated by flow cytometry or molecular techniques (BCL-2 detection). A minority of patients may present with a WBC count $>40 \times 10^9$/L) and extensive disease, generalised lymphadenopathy, hepatomegaly and a prominent splenomegaly. Usually, the circulating cells are small, with almost no visible cytoplasm, the nuclear chromatin is smooth without clumps of heterochromatin and no visible nucleolus, and the nuclear shape is angular and has a small cleft (Figure 23.12). Lymph node histology is essential for confirming a diagnosis of follicular lymphoma. Patients with high WBC and a marked proportion of lymphoma cells in blood have a poorer prognosis than those with a minor degree of spillover. Late-stage follicular lymphoma is characterised by multiple genetic lesions and may have circulating blasts (centroblasts); this corresponds with histological transformation to a large-cell lymphoma or the so-called blastic form of follicular lymphoma. The immunophenotypic characteristics of follicular lymphoma cells are shown in Table 27.2.

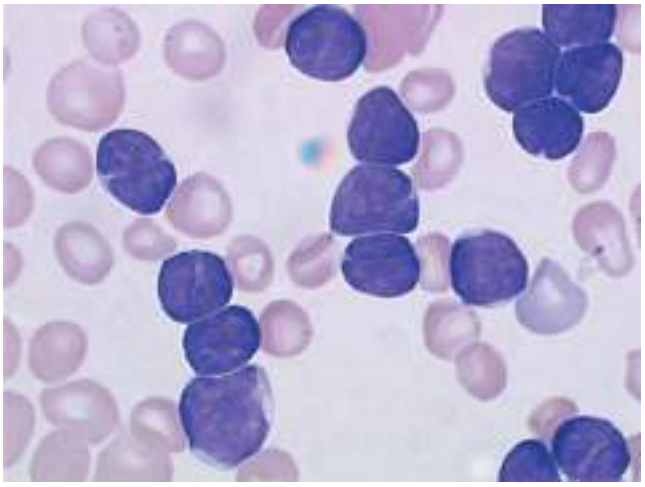


Figure 23.12 Blood film of a patient with follicular lymphoma presenting with significant lymphocytosis. The cells show a cleaved nucleus, angular shape, homogeneous chromatin pattern and high nucleus-to-cytoplasm ratio and small size.

Splenic B-cell lymphoma/leukaemia with prominent nucleoli (Hairy-cell leukaemia variant)

The so-called HCL variant (HCLv was included in the WHO classification (2017) as a provisional entity under the umbrella of splenic lymphomas. In the 5th Edition of the WHO classification (2022), HCLv falls under the term 'Splenic B-cell lymphoma/leukaemia with prominent nucleoli'. It affects older people, predominantly males and is characterised by the presence in peripheral blood of a large proportion of small to medium-sized villous lymphoid cells. The abnormal lymphoid cells have a polar distribution of the villi, and the nucleolus is small or not visible (Figure 23.13) The neoplastic cells in HCLv show intense expression of pan-B-cell markers, such as CD19, CD20, CD22 and CD79b, and also brightly positivity for surface light chains. CD11c, CD103 and, rarely, CD123 may be positive. CD25, CD200 and Annexin A1 are not expressed. The MCV is normal and there is no monocytopenia. Del (17p)/*TP53* mutation and del(11q)/*ATM* mutation may be present. In contrast to HCL, no *BRAF* V600E mutation is present. *MAP2K1* is the most frequently mutated gene, encoding *MAP2K1/MEK1* downstream from *BRAF*. HCLv frequently uses IGHV4-34 in unmutated conformation (approximately, 40% of cases), which is correlated with a higher disease burden. The WBC count can be high (e.g. $50–80 \times 10^9$/L). In contrast to HCL, the bone marrow aspirate is usually cellular and the bone marrow trephine shows a mixed pattern of predominantly interstitial and to a lesser extent, intrasinusoidal infiltration by leukaemic cells. HCL responds poorly to purine analogues given as single agents. For this reason, it is recommended that cladribine plus rituximab be used as first-line therapy. Small numbers of patients with HCLv have been treated with moxetumumab pasudotox (an anti-CD22 immunotoxin) with inferior results than seen in HCLv, which may be due to the

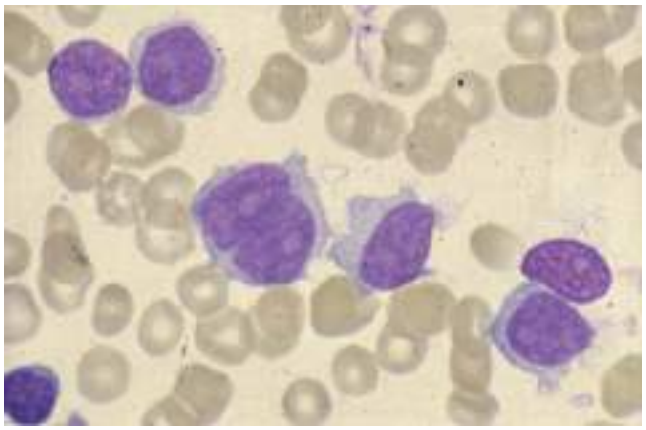


Figure 23.13 Blood film from a case of HCL variant showing large nucleolated villous cells, one of them binucleated.

presence of bulky disease. Trametinib (a MEK inhibitor) has been shown to have treatment activity. Splenectomy may be considered in refractory cases to improve symptoms and cytopenias. BTKi and BCL2i are emerging as treatment possibilities.

Splenic diffuse red pulp small B-cell lymphoma

The SDRPL is characterised by the presence of a large proportion of small-to-medium-sized lymphoid cells in peripheral blood with clumped chromatin and round nuclear outline and villous cytoplasm. The spleen shows a diffuse pattern of infiltration by monomorphous small cells with small round nuclei and pale cytoplasm. Bone marrow shows intrasinusoidal infiltration. The monoclonal B cells express CD11c, CD103 and CD123; CD25 and Annexin D1 are not expressed. This disease is more common in older men and usually presents with splenomegaly and moderate lymphocytosis. The differential diagnosis from other splenic lymphomas, such as SMZL can be difficult because of their similar clinical presentations and the absence of specific molecular markers. The diagnosis may eventually require the examination of the spleen histology. Splenectomy may be useful to control the disease.

Acknowledgements

This chapter is dedicated to the memory of the late Professor Daniel Catovsky who authored it in past editions. Thanks are due to Stefano Molica for helpful comments on novel CLL therapies. The author wishes to apologise to those colleagues whose contributions are referred to in this chapter but could not be quoted because of space limitations.

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CHAPTER 24

T-cell lymphoma and lymphoproliferative disorders

24

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Introduction

Chronic T-cell lymphoproliferative disorders are a group of neoplastic diseases that result from the clonal proliferation of mature post-thymic T-lymphocytes, being therefore clearly separated from diseases arising from T-cell (prethymic or thymus-derived) precursors, such as T-cell acute lymphoblastic leukaemia and lymphoblastic lymphoma.

The World Health Organization (WHO) classification provides clear definitions for the diagnosis of the major subtypes of T-cell lymphoproliferative disorders, which are classified on the basis of their clinical behaviour (indolent versus aggressive) and presentation (nodal, extranodal or leukaemic). In 2022, the classification of haematologic and lymphoid neoplasm was updated, now recognising 34 established entities of mature T-cell non-Hodgkin lymphomas and lymphoproliferative diseases. The classification underscores the great heterogeneity of T-cell neoplasms on account of morphological, immunophenotypical, clinical and molecular features of each disease entity. Furthermore, it provides the tools to better stratify patients in terms of prognosis and outcomes and to guide decisions before and throughout treatment. Table 24.1 reports the most recent WHO classification of mature T-cell neoplasms.

Mycosis fungoides and primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders represent extranodal (cutaneous) diseases with a prolonged natural history; conversely, anaplastic large-cell lymphoma, angioimmunoblastic T-cell lymphoma and peripheral T-cell non-Hodgkin lymphoma, not otherwise specified, to cite the most relevant, represent the aggressive nodal counterpart. Enteropathy-associated T-cell lymphoma and hepatosplenic T-cell lymphoma have an extranodal presentation and usually behave aggressively, as well as some rarer cutaneous T-cell lymphoma entities, such as primary cutaneous aggressive epidermotropic CD8$^+$ T-cell lymphoma and cutaneous γ/δ T-cell lymphoma. T-prolymphocytic leukaemia and large granular lymphocyte leukaemia are indeed diseases with a primary leukaemic presentation, as they arise in the bone marrow and then evolve with a peripheral blood leukaemic presentation. Finally, Sézary syndrome may be considered as the leukaemic phase of a T-cell cutaneous lymphoma, since the tumour arises in an extranodal organ – i.e. the skin – but also displays a characteristic leukaemic picture.

This chapter focuses on the most significant clinical entities, by grouping together chronic T-cell leukaemias, peripheral T-cell non-Hodgkin lymphomas and cutaneous T-cell non-Hodgkin lymphomas, and provides the description of disease features and morphology, the basis for their diagnosis and the therapeutic approaches, with a closer look at new molecules and innovative treatment strategies.

Chronic T-cell leukaemias

Two distinct chronic T-cell lymphoproliferative disorders present with a primary leukaemic phase: T-prolymphocytic leukaemia (T-PLL) and large granular lymphocyte (LGL) leukaemia.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 24.1 The 2022 World Health Organisation (WHO) classification of mature T- and NK-cell neoplasms. Highlighted entities in bold are explained in the text.

Mature T/NK leukaemias	**T prolymphocytic leukaemia**
	T large granular lymphocytic leukaemia
	NK large granular lymphocytic leukaemia
	Adult T-cell leukaemia/lymphoma
	Sézary syndrome
	Aggressive NK-cell leukaemia
Primary cutaneous T-cell lymphoma	Primary cutaneous $CD4^+$ small/medium T-cell lymphoproliferative disorder
	Primary cutaneous acral $CD8^+$ lymphoproliferative disorder
	Mycosis fungoides
	Primary cutaneous $CD30^+$ T-cell lymphoproliferative disorder – Lymphomatoid papulosis
	Primary cutaneous $CD30^+$ T-cell lymphoproliferative disorder – Primary cutaneous anaplastic large cell lymphoma
	Subcutaneous panniculitis-like T-cell lymphoma
	Primary cutaneous γ/δ T-cell lymphoma
	Primary cutaneous $CD8^+$ aggressive epidermotropic cytotoxic T-cell lymphoma
	Primary cutaneous peripheral T-cell lymphoma, NOS
Peripheral T-cell lymphoma	Indolent T-cell lymphoma of the GI tract
	Indolent NK-cell lymphoproliferative disorder of the GI tract
Extranodal types	**Enteropathy-associated T-cell lymphoma**
	Monomorphic epitheliotropic intestinal T-cell lymphoma
	Intestinal T-cell lymphoma, NOS
	Hepatosplenic T-cell lymphoma
Peripheral T-cell lymphoma	**ALK-positive anaplastic large-cell lymphoma**
	ALK-negative anaplastic large-cell lymphoma
Nodal types	**Breast implant-associated anaplastic large-cell lymphoma**
	Nodal TFH cell lymphoma, angioimmunoblastic-type
	Nodal TFH cell lymphoma, follicular-type
	Nodal TFH cell lymphoma, NOS
	Peripheral T-cell lymphoma, NOS
EBV^+ NK/T-cell lymphomas and lymphoid proliferations	EBV^+ nodal T- and NK-cell lymphoma
	Extranodal NK/T-cell lymphoma
	Severe mosquito bite allergy
	Hydroa vacciniforme lymphoproliferative disorder
	Systemic chronic active EBV disease
	Systemic EBV^+ T-cell lymphoma of childhood

NOS, not otherwise specified; GI, gastrointestinal; TFH, T-follicular helper; EBV, Epstein-Barr virus.

T-prolymphocytic leukaemia

T-PLL is a rare, post-thymic, mature T-cell neoplasm, accounting for 2% of mature lymphoid leukaemias. It generally displays an aggressive clinical course, with only a minority of patients being asymptomatic at presentation. Disease progression may occur rapidly, even in asymptomatic patients. Older adults are generally affected (median age of 61 years), with a male predominance.

Clinical features

Splenomegaly, seen in almost two-thirds of patients, and marked peripheral blood lymphocytosis, with circulating prolymphocytes, are distinctive clinical aspects. Hepatomegaly and lymphadenopathies may be seen in up to half of patients, as well as skin nodules, maculopapular rash or erythroderma. Peripheral oedema (periorbital or conjunctival), pleuroperitoneal effusions or central nervous system involvement may also be present, especially during disease progression. Anaemia and thrombocytopenia are usually documented, along with a lactate-dehydrogenase elevation.

Diagnosis

Diagnosis is established on morphological, immunophenotyping and cytogenetic tests, as outlined in Table 24.2. All the three major criteria need to be fulfilled for the diagnosis, or alternatively the first two major criteria and one minor criterion.

Table 24.2 Requirements to establish the diagnosis of T-PLL.

Major criteria	Minor criteria
– More than 5×10^9/L cells of T-PLL phenotype in peripheral blood or bone marrow. – T-cell clonality (by polymerase chain reaction for *TCRB/TCRG*, or by flow cytometry). – Abnormalities of 14q32 or Xq28 or expression of *TCL1A/B* or *MTCP1*.	– Abnormalities involving chromosome 11 (11q22.3; *ATM*). – Abnormalities in chromosome 8. – Abnormalities in chromosomes 5, 12, 13, 22 or complex karyotype. – Involvement of T-PLL specific site (splenomegaly, effusions).

Prolymphocytes are medium-sized cells, displaying a high nuclear-to-cytoplasm ratio, with round, oval, irregular or convoluted nuclei and a single prominent nucleolus; the cytoplasm is agranular, with blebs (Figure 24.1). In up to 20% of cases, prolymphocytes may resemble small lymphocytes or show a cerebriform nuclear appearance. The bone marrow is invariably involved, with a diffuse and interstitial pattern, as well as the spleen, which shows an expansion of both red and white pulp. Tissue histology, however, is not mandatory for diagnosis. Immunophenotyping by flow cytometry demonstrates a positivity for CD7 (strong), CD5 and CD2, with lack of expression of terminal deoxynucleotidyl transferase (TdT) and CD1a. CD52 is expressed at high density. T-cell receptor α/β genes are rearranged in most of the cases, and just in a minority of instances a γ/δ rearrangement is detected. In virtually all cases, the Xq28 (*MTCP1* locus) or 14q32.1 (*TCL1* and *TCL1b* loci) regions are involved in translocations or inversions with *TCRA/D* at 14q11. The demonstration of either cytogenetic abnormality is a major criterion for diagnosis. Complex karyotype alterations or abnormalities involving chromosomes 8, 11, 5, 12, 13 and 22 may also be detected.

Therapy

Although in rare asymptomatic cases, an active surveillance programme may be pursued, systemic treatment is usually needed sooner rather than later due to the clinically aggressive and rapid natural history of the disease. More precisely, any the following are indications to treat: constitutional symptoms (fatigue, unintentional weight loss, drenching night sweats or fever without signs of infection), signs of bone marrow failure (haemoglobin <100 g/L, platelets $<100 \times 10^9$/L), rapidly enlarging nodes, spleen or liver, increasing lymphocytosis (doubling-time shorter than 6 months), extranodal involvement (including effusions or central nervous system involvement).

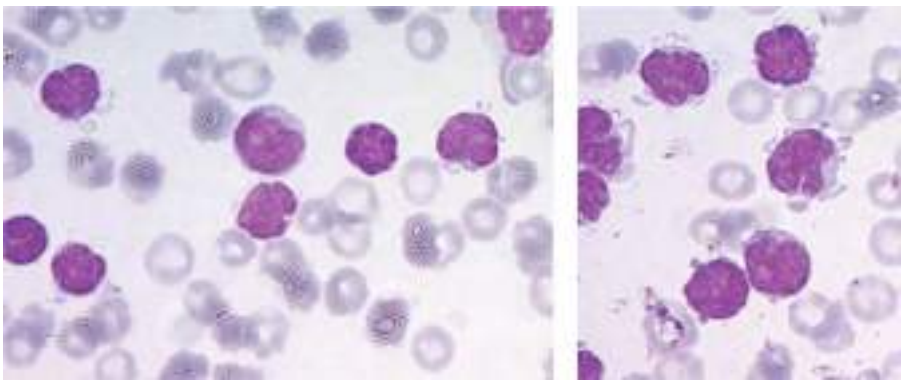
Intravenous alemtuzumab, a humanised anti-CD52 antibody, is the first-line treatment of choice, yielding up to an 80% of complete response in treatment-naïve patients. Although prolonged responses to alemtuzumab have been documented, all patients do eventually relapse: therefore, a consolidation with an allogeneic stem cell transplantation in first remission may offer a chance for a better long-term survival. Autologous stem-cell transplantation may also be beneficial for patients, but it does not result in a cure. Re-treatment with alemtuzumab at disease relapse is feasible if remission duration is longer than 6 months. Purine analogues, such as pentostatin, cladribine, nelarabine or fludarabine (alone or in combination with alemtuzumab) may be a reasonable treatment alternative, with some evidence of activity in T-PLL.

Prognosis

Response to alemtuzumab is the main outcome predictor: non-responders have a median survival of only 4 months. Five-year overall survival is only approximately 20%, with high white cell counts, older age and short lymphocyte doubling time being adverse prognostic factors.

Large granular lymphocyte leukaemia

LGL leukaemia is characterised by the clonal expansion of large granular lymphocytes, both of T (T-LGL) or natural killer (NKLGL) origin. The 2022 WHO classification includes T-LGL



(a) (b)

Figure 24.1 Peripheral blood smear showing T-prolymphocytes. (a) Nuclei are round to markedly irregular (indented, cerebriform), the chromatin is clumped, with inconspicuous nucleoli. (b) Blebs and cytoplasm protrusions are easily seen.

in the subgroup of mature peripheral T-cell and NK-cell neoplasms, along with a clinically distinct disease, which is the aggressive NK-cell leukaemia.

The frequency of the disease has never been accurately established; however, it ranges from 2 to 6% of the chronic lymphoproliferative disorders, being slightly more frequent in Asia than in North America and Europe. Men and women are equally affected, at a median age of 60 (range 12–87) years, with less than 25% of patients being younger than 50 years. Roughly 85% of LGL cases are of T-cell origin, while nearly 10% are described as chronic NK-LGL leukaemia. The aggressive NK-cell leukaemia is mostly reported in people of Asian origin, displaying a very poor outcome and a significantly high rate of treatment refractoriness.

The aetiology remains unclear, although a prolonged response to an antigenic (possibly viral) stimulus may be hypothesised. An initial oligoclonal proliferation of LGLs may lead to the emergence of a prevalent clone if the inciting antigenic drive persists due in part to the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Somatic mutations, particularly of *STAT3*, accelerate the clonal expansion and evolution of the leukaemic cells and lead to the production of pro-inflammatory cytokines that contribute to the development of cytopenias and autoimmune phenomena. Resistance of LGLs to apoptosis is also a key pathogenetic mechanism, which is mediated by the enhanced expression of the antiapoptotic molecule Mcl-1, the increased levels of IL-15 and the high expression of Fas and Fas-ligand (FasL) on neoplastic cells.

Clinical features

The disease displays a chronic and indolent course, although up to two-thirds of patients are symptomatic at diagnosis. Clinically relevant aspects are similar for both T- and NK-LGL and are represented by peripheral cytopenias, namely neutropenia and anaemia. Severe neutropenia can be asymptomatic for very long periods of time, albeit some patients show oral aphthous ulcerations or fever due to bacterial infections. Anaemia may be severe, due to multiple mechanisms, including autoimmune haemolysis and pure red cell aplasia, and may require transfusion support. Splenomegaly is reported in up to 50% of cases, without hepatomegaly or lymphadenopathy.

Rheumatoid arthritis is the most commonly associated autoimmune manifestation, in 11–36% of patients. A positive rheumatoid factor and the detection of antinuclear antibodies (along with polyclonal hypergammaglobulinaemia) is seen in 40–60% of cases, less frequently observed in NK-LGL. Less common autoimmune diseases include Sjögren syndrome, systemic lupus erythematosus, Behçet disease, autoimmune thyroid disorders and autoimmune thrombocytopenia.

Lymphocytosis ($>4\times10^9$/L) is seen in more than half of patients, with mean circulating LGLs varying from 0.5 to 10×10^9/L. Bone marrow involvement is documented in more than 70% of cases, without any linear correlation between the extent of marrow infiltration and the severity of symptoms or cytopenias.

Diagnosis

Diagnosis is established once an increased number of clonal LGLs is documented in a compatible clinical context, both morphologically and by immunophenotyping. LGLs are larger than quiescent lymphocytes and show an abundant cytoplasm with azurophilic granules and a reniform or round nucleus. A peripheral count of $>2\times10^9$/L LGLs is a diagnostic criterion; in case of lower LGL counts, a bone marrow biopsy (showing interstitial infiltration with linear arrays of CD8, granzyme B, perforin, TIA-1 positivity) is recommended to confirm the diagnosis. T-LGLs are CD3+, CD8+, CD57+, $CD5^{dim}$ and CD45RA+, and show evidence of clonal T-cell receptor γ gene rearrangement (which makes the distinction from reactive LGL proliferation). On the other hand, NK-LGLs are CD3–, CD2+, CD4–, CD8+, CD16+, CD56+ and CD57±.

Therapy

Therapy should be instituted in case of severe neutropenia (neutrophils $<0.5\times10^9$/L), recurrent infections (regardless of the absolute neutrophil count), symptomatic or transfusion-dependent anaemia or associated immune diseases (mainly rheumatoid arthritis). No standard treatment exists; however, immunosuppressive treatments represent the mainstay of therapy. Single-agent methotrexate (10 mg/m^2/week, orally) is adequate for patients with neutropenia and arthritis, to be administered for at least 4 months before response assessment and to be continued as long as the patient responds. For those with anaemia or pure red cell aplasia, oral cyclophosphamide at a daily dose of 50–100 mg for at least 4 months (up to 12 months in responding patients) is the treatment of choice, comparing favourably with methotrexate in terms of response rates (66 and 55%, respectively). Cyclosporine A (3 mg/kg/day) is an alternative first-line therapy in patients with anaemia and a suitable second-line, to be maintained indefinitely, as long as it is tolerated. Recent data suggests that Jak inhibitors are very active in cases which contain an activating *STAT3* mutation.

Prognosis

LGL leukaemia generally behaves indolently; it is difficult to evaluate the proportion of patients who will require a systemic therapy due to the worsening of cytopenias or the appearance of symptoms (33–80% in reported series). Aggressive NK disease, with high LGL peripheral counts and marrow infiltrate, massive organomegaly and lymphoma-related B symptoms display a poor prognosis, and patients appear refractory to any treatment.

Nodal peripheral T-cell non-Hodgkin lymphomas

Peripheral T-cell non-Hodgkin lymphomas (PTCL) are a distinct form of rare chronic T-cell lymphoproliferative disease, clearly separate from cutaneous T-cell lymphomas (CTCL), discussed below. The incidence of PTCL in the United States is less than 1 case per 100,000 people; worldwide, the prevalence of

each specific disease entity profoundly depends on the geographic region, although the biological reason for the observed discrepancies is still largely unknown. In Western countries, PTCL accounts for 15–20% of aggressive lymphomas, and 5–10% of all non-Hodgkin lymphomas. In Asia, however, incidence is higher, and 15–20% of all lymphomas are classified as PTCL or NK/T-cell lymphoma. PTCL-not otherwise specified (NOS) is the most common reported subtype in both North America and Europe, whereas NK/T-cell lymphoma and adult T-cell leukaemia/lymphoma are more common in Asia, the latter being specifically represented in Japan. Anaplastic large-cell lymphoma (ALCL) is more represented in the United States, whereas angioimmunoblastic T-cell lymphoma (AITL) is more frequent in Europe than in Asia or in North America.

In the context of the 2022 WHO classification, PTCL represent a group of mature T-cell aggressive diseases, primarily with a nodal origin (PTCL-NOS, ALCL, AITL being the most clinically relevant entities), although extranodal subtypes do exist – being far less common – described by their tissue tropism (e.g. enteropathy-associated T-cell lymphoma and hepatosplenic T-cell lymphoma).

Diagnostic accuracy amongst expert haematopathologists varies according to subtype. For example, diagnostic accuracy is very good (>97% agreement amongst experts) for anaplastic lymphoma kinase-positive ALCL, but poor (<85%) for the other lymphoma subtypes, being only 75% for the most common subtype, PTCL-NOS. Importantly, several cases previously characterised as PTCL-NOS can be reclassified into more recent WHO-recognised categories, such as AITL or ALCL. This underscores the role of recent biologic insights into the pathogenesis of these diseases, as well as more specific diagnostic markers and diagnostic criteria in enhancing the diagnostic accuracy and reproducibility for these entities.

PTCL are staged according to the Ann Arbor staging system, with the same techniques used for B-cell lymphomas. The majority of PTCL patients, with the exception of ALCL, present with an advanced (III or IV) stage disease. Positron emission tomography (PET) scanning has proved its usefulness in the staging of PTCL, although maximum standard uptake values in patients with PTCL seem somewhat lower than in aggressive B-cell lymphomas, and generally less pronounced for extranodal lesion than for nodal areas of disease. Data regarding the role of post-treatment PET scan are still inconclusive, since negative scans may not be always associated with significantly improved clinical outcomes.

The International Prognostic Index (IPI) remains the mainstay of the prognostic stratification of patients with PTCL, being inversely related to survival in a manner similar to B-cell lymphomas, although overall survival in each category is lower.

Peripheral T-cell non-Hodgkin lymphoma, not otherwise specified

PTCL-NOS is the most common subtype, accounting for at least 25–30% of PTCL. However, it does not represent a single entity, since as many as 20% of cases have shown a gene expression profile characteristic of other PTCL subtypes, such as AITL, ALCL, extranodal NK/T-cell lymphoma or adult T-cell leukaemia/lymphoma, sometimes with molecular markers suggesting a peculiar T-follicular helper (T_{FH}) origin. Moreover, another subgroup shows features of cytotoxic T-cells, thus suggesting a separate disease entity, with a worse prognosis than any other PTCL-NOS. The diagnosis of PTCL-NOS should then be established once other categories of PTCL have been excluded.

Clinical features

The disease tends to affect adult patients (median age at presentation is 60 years), with a male predominance. Children can also be affected. Nodal involvement is the most relevant feature at diagnosis, although any organ can be affected (including bone marrow, liver, spleen and skin). Advanced stage at presentation is relatively common (nearly 70% of cases); lymphoma-related symptoms, lactate-dehydrogenase elevation and poor performance status are documented in more than half of the cases; almost two-thirds of the patients present an intermediate to high IPI score.

Morphology and biology

Lymph node infiltration is generally diffuse, with neoplastic elements showing a variable morphology, ranging from small cells with irregular nuclei to large cells with prominent nucleoli and mitotic figures (Figure 24.2); cells with clear cytoplasm and occasionally Reed–Sternberg-like cells can also be encountered, along with an inflammatory background composed of small reactive lymphocytes, eosinophils, plasma cells and epithelioid histiocytes. A diffuse or interfollicular infiltrate of epithelioid histiocytes is particularly evident in the lymphoepithelioid (Lennert) variant. A subset of PTCL-NOS shows a vaguely nodular pattern, featuring a follicular lymphoma or a lymphocyte-predominant

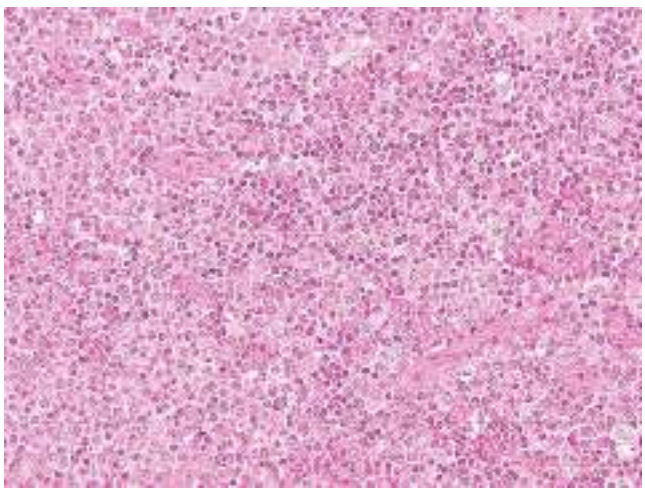


Figure 24.2 Peripheral T-cell lymphoma, not otherwise specified (haematoxylin and eosin stain, magnification 200×).

Hodgkin lymphoma pattern: such cases more often display a phenotype related to T_{FH} cells (as outlined in the paragraph on AITL). Neoplastic cells stain positively for CD2, CD3, CD5 and CD7, with a variable expression of CD4 and CD8; aberrant T-cell phenotypes, with lack of expression of some antigens, can also be observed. The CD30 antigen is variably expressed, mostly in large-cell variants. Rare cases of PTCL-NOS express the cytotoxic granule-associated proteins (perforin, granzyme B and TIA-1), specifically in the lymphoepithelioid variant.

Recently, gene expression profile studies have contributed to the dissection of PTCL-NOS into molecularly distinct entities, according to the overexpression of *GATA3* and *TBX21*. *GATA3* expression correlates with a worse prognosis (<20% of patients alive at 5 years), whereas cases with a *TBX21* signature have more favourable outcomes. Cases with a T_{FH} phenotype display recurrent genetic abnormalities in *TET2*, *IDH2*, *DNMT3A*, *RHOA* and *CD38*, as is seen with AITL: for this reason, they should be more correctly classified as nodal PTCL with a T_{FH} phenotype.

Prognosis

The IPI is significantly associated with treatment outcomes. Reported 5-year overall survival for IPI score 0/1 and 4/5 is 50 and 11% respectively with 5-year progression free survival of 33 and 6%. A newly introduced prognostic index specifically designed for PTCL (PIT) includes age, lactate-dehydrogenase elevation, performance status and bone marrow involvement, and better allows the stratification of patients into more specific prognostic groups, with a 5-year overall survival for the most favourable group (i.e. the one with no adverse factors) of 62, and of 18% for patients presenting with three or four adverse prognostic factors.

Angioimmunoblastic T-cell lymphoma

AITL makes up nearly 15–20% of cases of PTCL, being the second most common variety. AITL is a T_{FH}-derived neoplasm that originates from follicular helper T-lymphocytes: these cells are normally present in lymphoid follicles, being involved in the germinal centre reaction in close collaboration with B-lymphocytes. Given that a number of recurrent mutations and a peculiar gene signature characterise a significant proportion of AITL cases, as well as some cases of PTCL-NOS which manifest a T_{FH} phenotype (as said above), the 2022 WHO classification unifies under a common heading AITL, follicular T-cell lymphoma and nodal PTCL with T_{FH} phenotype.

Clinical features

The disease typically affects older adults (median age 65 years), and it is clinically characterised by an aggressive clinical course, an advanced stage at presentation (nearly 90% of patients show stage III/IV) and by the presence of lymphoma-related symptoms (in about 70% of patients since disease onset). Patients complain of a generalised lymphadenopathy, which is usually associated with hepatosplenomegaly and rash. Bone marrow is involved in 28% of cases. Lactate-dehydrogenase elevation, polyclonal hypergammaglobulinaemia and autoimmune manifestations (such as autoimmune haemolytic anaemia, cold agglutinin disease, rheumatoid factor positivity and anti-smooth-muscle antibody positivity) are frequently associated findings, as well as the presence of peripheral oedema, pleural or peritoneal effusions and joint pain.

Morphology and biology

From a histological point of view, the architecture of the lymph node appears completely effaced by a T-cell infiltrate, which extends beyond the node capsule, but characteristically sparing the subcapsular sinus, which appears dilated (Figure 24.3). The neoplastic cells show an aberrant expression of T-cell markers (CD2, CD3, CD5 and CD7) and display the phenotype of T_{FH} cells, with the positivity of at least three of the following markers: CD4, CD10, BCL-6, PD-1, CXCL13 and SAP. Reactive lymphocytes, eosinophils, plasma cells, follicular dendritic cells and histiocytes represent accompanying non-neoplastic populations, along with the presence of scattered large CD20+ immunoblastic cells, usually staining positively for EBV-encoded RNA (EBNA). A characteristic feature of AITL is a prominent vascular proliferation, as a consequence of the over-expression of the vascular endothelial growth factor (VEGF)-A gene, both in lymphoma and endothelial cells.

The AITL mutational landscape is complex: *TET2*, *IDH2*, *DNMT3A* and *RHOA* are amongst the most relevant mutated genes, possibly with a role in lymphomagenesis. *TET2* is mutated in half to three-quarters of the cases, along with *IDH2*; *RHOA* mutations can be found in up to 70% of the cases and occur with concomitant *TET2* mutations.

Prognosis

Rapid disease progression is seen in most patients, accompanied by frequent infectious complications, leading to a median survival of less than 3 years.

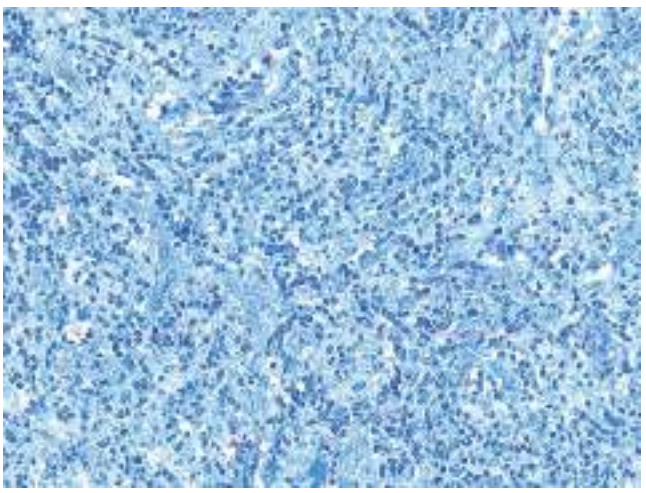


Figure 24.3 Angioimmunoblastic T-cell lymphoma (Giemsa stain, magnification 200×).

Anaplastic large-cell lymphoma

First described as a group of tumours displaying large cells with bizarre morphology, prominent sinusoidal invasion and Ki-1 antigen (nowadays recognised as CD30) expression, ALCL has been recognised as a distinct clinical entity since the third edition of the WHO classification, which grouped together a significant proportion of cases characterised by the expression of the anaplastic lymphoma kinase (ALK), and a smaller subcategory, lacking the expression of ALK (ALK-negative ALCL). When it became clear that ALK-positive ALCL represented a relatively homogeneous disease subtype, also displaying a better clinical course compared with ALK-negative ALCL, the two disease entities were clearly separated. The 2022 WHO classification recognises an ALK-positive ALCL and an ALK-negative ALCL, the latter being no longer conceived as a provisional subcategory but rather defined as a specific disease entity. These two entities display a markedly different prognosis when treated frontline with conventional therapy and are managed differently. The current WHO classification also recognises two more ALCL subcategories. The former has an exquisitely cutaneous presentation, thus defined primary cutaneous ALCL (pcALCL, discussed in the cutaneous T-cell lymphoma section). The latter is associated with breast prostheses and termed breast implant-associated (BIA) ALCL.

Clinical features

Nearly 70% of patients with ALCL present with an advanced stage, and the clinical scenario of ALK-positive and ALK-negative varieties is similar. Peripheral or abdominal nodal disease is associated in nearly one-third of patients with an extranodal involvement (especially in patients exhibiting the ALK-positive variant), with bone marrow, skeleton and lung being the most represented sites. BIA-ALCL mainly presents as seroma fluid between the breast implant and the fibrous capsule, with most of the cases being localised at onset. In a minority of cases, it develops as a growing mass, eventually with distant dissemination.

Biology and prognosis

In more than two-thirds of cases, the ALK protein is overexpressed, mainly due to the translocation of the *ALK* gene on chromosome 2 to the nucleolar phosphoprotein 1 (*NPM1*) gene on chromosome 5, most frequently as a result of the chromosomal translocation t(2;5)(p23;q35), although at least 11 variant translocations involving the *ALK* gene at 2p23 have been described. Patients with ALK-positive lymphoma are much younger (median age of 34 years) than those with the ALK-negative variant (median age of 58 years). The expression of the ALK protein makes a substantial prognostic difference in terms of overall survival and failure-free-survival, which both favour patients with ALK-positive ALCL compared with ALK-negative disease (5-year overall survival, 70 versus 49%, 5-year failure-free survival, 60 versus 36%). However, patients aged over 40 years tend to display a similar outcome in terms of survival, independently from the expression of the ALK protein.

The molecular biology of ALK-negative ALCL is far less known: in nearly one-third of cases, rearrangements of *DUSP22* can be found identifying a subset of patients with a more favourable outcome. Conversely, rearrangements of the *TP63* gene are associated with much worse outcomes.

Morphology

ALCL exhibits a wide range of cytological appearances, with no distinction on morphological grounds between the ALK-positive and the ALK-negative variants (Figure 24.4). Different morphological variants of ALK-positive ALCL are described: the common pattern is composed predominantly of pleomorphic large cells, with irregular, eccentric, kidney-shaped nuclei (hallmark cells); the normal lymph node architecture is frequently obliterated and an intrasinusoidal cell growth is often documented, mimicking a metastatic malignancy. The lymphohistiocytic pattern is characterised by the presence of tumour cells admixed with a variable amount of histiocytes, whereas the small cell pattern shows a predominance of small- to medium-sized neoplastic cells, with round or irregular nuclei and sometimes with a clear cytoplasm. The Hodgkin-like pattern has morphologic features

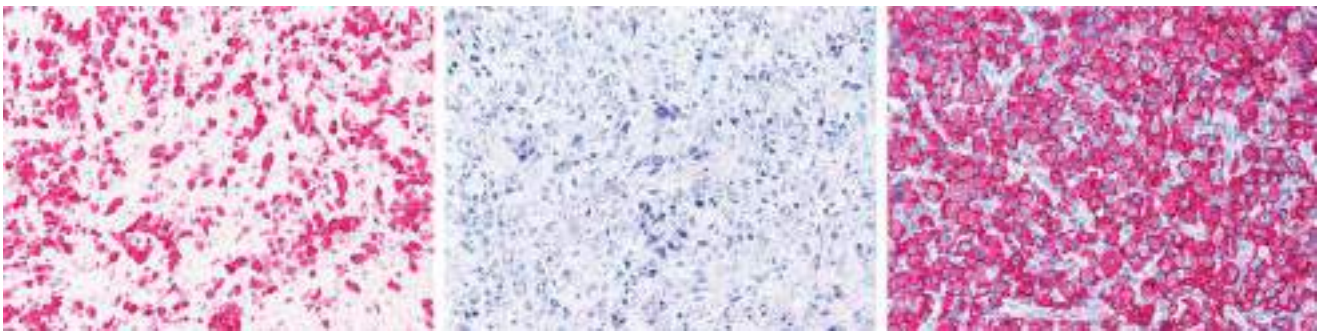


Figure 24.4 Anaplastic large-cell lymphoma (ALCL). (a) ALK-positive ALCL (immunohistochemistry for ALK protein, magnification 200×). (b) ALK-negative ALCL (Giemsa stain, magnification 200×). (c) ALK-negative ALCL (immunohistochemistry for CD30, magnification 200×).

mimicking nodular sclerosis classic Hodgkin lymphoma, with a significant number of tumour cells resembling Reed–Sternberg cells. Composite patterns may be seen in up to 20% of cases since features of more than one previously described pattern can be appreciated within a single lymph node specimen. The ALK-negative ALCL mostly shows a common type pattern of growth.

All ALCL are – by definition – positive for CD30, which is expressed on the cell membrane and in the Golgi region, predominantly in larger tumour cells. The great majority of ALCL cases express T-cell or NK-cell antigens, although several pan-T-cell antigens may be lost or unexpressed, yielding to an apparently T-null phenotype. The ALK staining may be cytoplasmic, nuclear or nucleolar, or it may be restricted to either the cytoplasm or the cell membrane. ALK expression is absent from all normal postnatal human tissues, except some rare cells in the brain although can be seen in some forms of lung cancer.

Extranodal peripheral T-cell non-Hodgkin lymphomas

Primary intestinal T-cell lymphomas

Enteropathy-associated T-cell lymphoma (EATL) is an aggressive disorder, frequently associated with coeliac disease; its incidence varies geographically, and it overlaps that of gluten-sensitive enteropathy, although not all patients with EATL have an overt coeliac sprue. EATL is most frequent in the sixth decade of life, with a similar incidence amongst men and women. It usually presents with abdominal pain, fatigue and anorexia, as well as with an exacerbation of typical symptoms of uncontrolled coeliac disease, such as diarrhoea and weight loss, together with hypoalbuminaemia and anaemia. Gastrointestinal ulceration, perforation, obstruction or haemorrhage may be frequent complications. The jejunum is mainly affected, although disease extension may be multifocal, also involving other intestinal segments.

Patients with EATL have a poor prognosis, one of the poorest of any subtype of PTCL, with a median 5-year overall survival of 20% and a progression-free survival of only 4%, which also remains dismal for those patients presenting with a more favourable IPI risk. Many patients, in fact, are unable to tolerate an aggressive chemotherapy approach.

Monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) is the second most common primary intestinal T-cell lymphoma. It occurs in older adults, at a median age of 61 years and with male predominance. In contrast to EATL, there is no definitive association with coeliac disease. The jejunum and ileum are the most commonly affected gastrointestinal segments, followed by the colon and the stomach. Extra-intestinal dissemination to mesenteric nodes or pelvic organs may also occur.

Nearly half of MEITL patients are asymptomatic, while a few shows gastrointestinal symptoms such as abdominal pain, weight loss, nausea or vomiting and diarrhoea. In some cases, the disease may have an acute onset due to intestinal perforation.

Hepatosplenic T-cell lymphoma

This subtype accounts for nearly 1.4% of PTCL, being more frequent in young men, who present with systemic symptoms, mainly fever, hepatic and splenic enlargement, along with peripheral cytopenias. It has been reported in the context of immune system disorders or alterations, including Crohn disease and solid organ transplantation. The tumour spreads to the spleen, the liver and the bone marrow, and infiltrates organs without forming growing and coalescent nodal masses.

The present treatments are largely unsatisfactory, and this disease displays a very poor prognosis, with a 5-year overall survival of 7% and progression-free survival of 0% although cures have been reported with early allogeneic stem cell transplantation.

Treatment of peripheral T-cell non-Hodgkin lymphomas

So far, the treatment strategies most widely applied in patients with PTCL are mainly derived from B-cell lymphomas. However, PTCL are biologically and clinically distinct from their B-cell counterparts, thus explaining the extremely different ranges of response to various therapies and the huge prognostic gap that exists between lymphomas of B or T origin.

The treatment approach for PTCL is discussed in this section. A chemotherapy-based first-line treatment is aimed at inducing a first clinical (complete) remission, which may be further consolidated with autologous or allogeneic stem cell transplantation (ASCT), depending on the specific PTCL subtype and IPI score at presentation. Although ASCT is frequently offered for consolidation of the first remission, its role is still a matter of discussion, as there is no randomised trial evidence of benefit.

Novel approaches are under consideration; on the one hand, to offer an alternative to those patients proving to be refractory after induction or relapsing after an autologous stem cell transplantation; on the other, to provide innovative treatment strategies to enhance the traditionally applied anthracycline-based induction schemes.

Induction chemotherapy

The cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) regimen, administered every 21 days, or CHOP-like schemes, still represent the backbone of induction therapy and are still the most widely used worldwide. No randomised clinical trials are able to prove; however, its superiority over different treatment schemes. ALCL patients are those who best respond to CHOP and CHOP-like regimens, in comparison to other PTCL subtypes, with 5-year overall response rates higher than 60%. AITL patients have a 5-year overall survival of 32% and a failure-free survival of 18% when treated with CHOP, and in patients with PTCL-NOS there is no clear evidence that the use of an anthracycline-containing regimen during induction does any better compared with an anthracycline-free first-line therapy. Of

note, nearly 40% of patients with ALK-positive disease and more than 60% of those with an ALK-negative ALCL fail to respond to therapy, and taken as a whole, PTCL display 5-year overall survival rates lower than 40% when treated first-line with CHOP and CHOP-like strategies. There is some evidence that the combination of CHOP plus etoposide (CHOEP) can be more effective than CHOP alone in PTCL-NOS and ALCL, mainly in younger patients with no lactate-dehydrogenase elevation.

Autologous stem cell transplantation in first remission

Given that patients responding to frontline induction chemotherapy generally display a short duration of response and a high risk of relapse, ASCT can be considered a suitable consolidative strategy for patients in first complete remission and with chemosensitive disease, especially for those with intermediate to high IPI scores at disease presentation and with histologies other than ALK-positive ALCL. Unfortunately, many patients are older and some tend to progress when they are still on induction therapy, thus ASCT is not appropriate in many cases. A dose-dense approach followed by up-front ASCT has demonstrated a 5-year overall and progression-free survival of 51 and 44%, respectively, across a variety of PTCL, with the best results seen in ALCL, even in the ALK-negative subtype. Disease status at transplantation is a major predictor of success.

Recently, registry studies have collectively failed to demonstrate a clear survival advantage of ASCT over no further treatment after induction if patients achieve a complete response. No randomised trials have been designed to clarify whether upfront ASCT should be regarded superior to conventional chemotherapy only or if patients in complete response after induction strictly require a high-dose chemotherapy consolidation. Due to lack of conclusive results on this issue, upfront ASCT is still recommended by current guidelines for patients with chemosensitive PTCL-NOS, AITL and other rarer subtypes.

Treatment of relapsed and refractory disease

A variety of new agents have been investigated in relapsed or refractory PTCL patients, and some of them have obtained approval for the treatment of patients in such a disease context.

Four compounds are licensed by the Food and Drug Administration (FDA) in the United States for their specific use as single agents: pralatrexate, a novel antifolate agent; romidepsin and belinostat, both histone deacetylase (HDAC) inhibitors; and brentuximab vedotin (BV), an anti-CD30 drug-conjugated monoclonal antibody. The approval in Europe of both pralatrexate and romidepsin was rejected due to lack of evident clinical benefit and belinostat is not licensed in Europe, but it has been granted orphan designation status by the European Medicines Agency. All of these agents are effective in a minority of patients in the relapsed and refractory setting but have failed to impact survival. BV, however, has been granted approval for the treatment of relapsed and refractory systemic ALCL: in a phase 2 trial, it produced an objective response rate of 85%, with a complete response in 57% of the cases and 5-year overall survival and progression-free survival rates of 60 and 39%, respectively. Some activity, although under an off-label use, has also been demonstrated in PTCL-NOS and ALCL. BV is now being moved to frontline PTCL treatment in combination with chemotherapy according to the results of the ECHELON-2 trial (see next paragraph).

Besides approved agents, lenalidomide, bendamustine and gemcitabine have demonstrated efficacy in various subtypes of PTCL after failure of one or more previous treatments, with response rate closely resembling those of FDA-approved compounds.

Innovative frontline combinations

The standard CHOP regimen has been used as a backbone for new frontline combinations with drugs that have demonstrated efficacy when used as single agents in relapsed or refractory patients (see above). Theoretically, frontline combinations may improve complete response rates leading to a higher proportion of patients to receiving ASCT. Phase 1/2 trials combining pralatrexate, romidepsin, belinostat and the anti-CD52 monoclonal antibody alemtuzumab with CHOP and CHOP-like regimens have shown the feasibility of the proposed combinations, but have failed to show the superiority of any combination regimens over CHOP itself, both in terms of disease control and survival.

The only clearly positive study was ECHELON-2, a large double-blind multicentre randomised phase 3 trial that tested the combination of BV plus cyclophosphamide, doxorubicin and prednisone (BV-CHP regimen) versus standard CHOP in previously untreated subjects with CD30-expressing PTCL (at least 10% of the cells). 70% of patients had systemic anaplastic large cell lymphoma with the study design deliberately enriching for this cohort. The overall response rate was 83% in the experimental arm and 72% in the experimental arm, with a complete response in 68 and 56% of cases, respectively. The 3-year PFS was 57% for the BV-CHP arm and 44% for the CHOP arm, and the difference remained significant irrespective of any consolidative ASCT. This also translated into a significantly prolonged overall survival. Based on these results, BV was approved in November 2018 by FDA for the treatment of adult patients with previously untreated systemic anaplastic large cell lymphoma or other CD30-positive PTCL in combination with chemotherapy. In Europe, the licence is restricted to ALCL.

Allogeneic transplantation

The rationale behind allogeneic stem cell transplantation rests on both the fact that allogeneic haematopoietic stem cells are free of tumour contamination and that a graft-versus-lymphoma effect can be hypothesised since donor-derived immune cells

are potentially capable of mediating an anti-tumour effect. Once again, disease status at transplantation and chemosensitivity are outcome predictors: a high percentage (almost 70%) of patients who are in remission at the time of transplantation can be cured of their disease, while only 25–30% of refractory patients may take advantage of this procedure. No differences have been documented in the relapse rates when a myeloablative conditioning is compared to reduced-intensity conditioning regimens, although a higher rate of non-relapse mortality with the myeloablative approach still exists.

New drugs

Patients failing an initial treatment are generally hard to treat effectively with conventional chemotherapy and constitute an unmet medical need. New agents targeting specific molecular hallmarks of these disease entities may have a rationale in the treatment of relapsed and refractory patients.

Demethylating agents, above all azacitidine, can play a role in patients with a T_{FH} PTCL, including AITL, given the presence of recurrent mutations in *TET2*, *DNMT3A* and *IDH2*. The anti JAK1/2 inhibitor ruxolitinib may also have a role in patients with T-cell neoplasms given the presence of mutations within the *JAK* and *STAT* system in T-cell neoplasms: results are very preliminary, but encouraging. Phosphatidyl-inositol 3-kinase (PI3K) inhibitors are also under evaluation in heavily pretreated patients: PI3K inhibitors block mitogen and prosurvival signals from the tumour and its microenvironment and in turn activate an anti-lymphoma immune response. Duvelisib, an oral dual inhibitor of PI3Kδ and PI3Kγ, was applied as single agent in a phase 1 trial involving both PTCL and CTCL patients, producing encouraging results in both cohorts and across multiple histologies, including PTCL-NOS, EATL and AITL. Checkpoint inhibitors that target and disrupt the interaction of PD-1 with its ligands (namely PD-L1) causing T-cell activation against the tumour have significantly improved the therapeutic landscape in several malignancies. In PTCL, an increased expression of PD-L1 has been described in both neoplastic and stromal cells across various subtypes: This supports the therapeutic usage of checkpoint inhibitors in this setting. Results are still very preliminary and further efforts are required to clarify the role of PD-1 inhibitors in PTCL. Chimeric antigen receptor (CAR) T-cells in PTCL are still under development. However, as pan-T cell aplasia induces severe immunodeficiency, the application of CAR T-cells in T-cell lymphoma patients is contrasted by the lack of specific antigens discriminating malignant *versus* normal T-lymphocytes.

Cutaneous T-cell non-Hodgkin lymphomas

Cutaneous T-cell lymphomas (CTCL) represent the vast majority (more than 75%) of primary cutaneous lymphomas, which are by definition largely confined to the skin at diagnosis, without significant involvement of lymph nodes or other extranodal organs. Mycosis fungoides and Sézary syndrome are the most relevant disease entities, together accounting for more than two-thirds of CTCL.

The incidence of CTCL has been increasing and the disease becomes more common with increasing age. The median age at onset is in the mid-50s, and a fourfold increase in patients over 70. The highest incidence rates are described in males and African–Americans.

Establishing a correct diagnosis is mandatory to define the best treatment strategy, both at disease onset and in a context of refractory disease. A multidisciplinary approach, with the involvement of clinical haemato-oncologists, experienced pathologists and dermatologists, is therefore recommended at every step of patient management. Several new agents have already proved their efficacy within recently conducted clinical trials, but many innovative drugs are now under investigation in order to expand the therapeutic options available for the treatment of these rare entities.

The current CTCL classification is the product of the collaboration of the WHO and the European Organisation for Research and Treatment of Cancer (EORTC) and is summarised in Table 24.3. Below is a description of the most frequent CTCL subtypes along with some therapeutic recommendations.

Table 24.3 World Health Organization/European Organisation for Research and Treatment of Cancer (WHO/EORTC) classification of primary cutaneous T-cell lymphomas. Provisional entities are listed in italics. Highlighted entities are discussed in detail in this chapter.

Mycosis fungoides
Mycosis fungoides, variants and subtypes
Folliculotropic mycosis fungoides
Pagetoid reticulosis
Granulomatous slack skin
Sézary syndrome
Adult T-cell leukaemia/lymphoma
Primary cutaneous CD30+ T-cell lymphoproliferative disorders
Primary cutaneous anaplastic large cell lymphoma
Lymphomatoid papulosis
Subcutaneous panniculitis-like T-cell lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Primary cutaneous peripheral T-cell lymphoma, rare subtypes
Primary cutaneous γ/δ T-cell lymphoma
Primary cutaneous aggressive CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous acral CD8+ T-cell lymphoma
Primary cutaneous CD4+ small/medium T-cell lymphoproliferative disorder
Primary cutaneous T-cell lymphoma, not otherwise specified

Mycosis fungoides

Mycosis fungoides represents the most frequent CTCL and consists of a mature neoplasm in which T-lymphocytes infiltrate the epidermis (early stages of the disease) or acquire the capacity to deeply proliferate and invade the dermis (plaques and tumour stages of the disease). It is a disease of the middle-aged and older adult.

Clinical features

The clinical history of this disease is characterised by an evolution over time in three different stages: the patch stage, the plaque stage and the tumour stage. The evolution reflects a progressively increased aggressiveness of the disease; it is associated with a different clinical appearance and prognosis, as well as with a particular treatment approach.

The patch stage is characterised by generally round to oval, flat or scaly cutaneous lesions – or patches – ranging from 1 to 10 cm in diameter, mainly appearing in areas of skin protected from sunlight (such as the buttocks, the groyne and the breasts, in women). Patches may present slight erythema, wrinkling or telangiectases; areas of hypo- or hyperpigmentation may occur (Figures 24.5 and 24.6). Patches may be confined to a limited skin area or may become disseminated. Patients at this stage are often asymptomatic, and this phase may behave indolently for several years or decades. Over time, cutaneous lesions may become infiltrated, and therefore flat or raised indurated lesions – or plaques – supervene. Plaques are red-to-brown in colour, often polycyclic and with a clear spot in the centre; sometimes they become ulcerated. An erythrodermic phase of the disease may be documented at either patch or plaque stage: the skin appears diffusely erythematous, infiltrated and desquamating (Figure 24.7); the face is also affected, although the

Figure 24.6 Folliculotropic mycosis fungoides.

Figure 24.5 Patches of mycosis fungoides.

Figure 24.7 Mycosis fungoides, erythrodermic phase.

Figure 24.8 Mycosis fungoides, tumour phase.

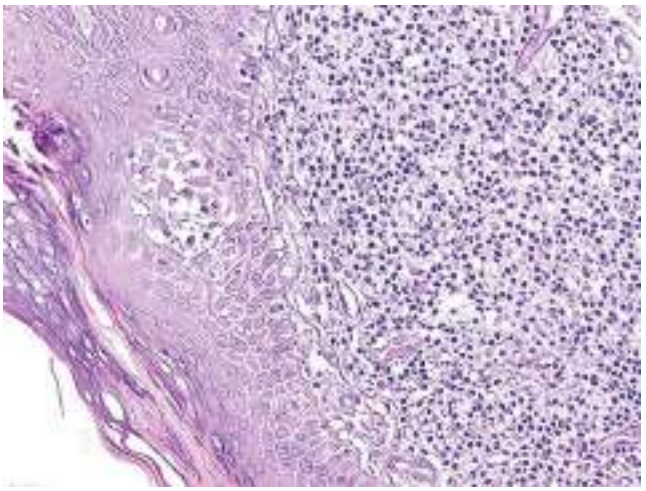


Figure 24.9 Histological appearance of plaques of mycosis fungoides (haematoxylin and eosin stain, magnification 200×).

elbow fold and the popliteal fossa seem to be characteristically spared. A thorough examination of peripheral blood is mandatory in this clinical context to correctly discriminate between an erythrodermic mycosis fungoides and Sézary syndrome. The tumour stage is characterised by the appearance of nodules or tumours on pre-existent patches or plaques (Figure 24.8): tumours are raised and plum coloured, with a smooth surface that usually ulcerates, and display a 'fungal' growth, hence the name of 'mycosis fungoides', first attributed by Alibert and Bazin to this disease. Along with the disease evolution to the tumour stage and the progressive appearance of new nodular lesions, the patient's general condition tends to worsen, and the prognosis becomes poor.

Morphology

The histopathologic appearance of the disease is extremely variable, depending on the stage. Early mycosis fungoides is not histologically recognisable with certainty: early patches feature small, isolated and scattered – or sometimes clustered – lymphocytes within the papillary dermis, with just a few within the epidermis. As infiltrates become denser, lymphocytes acquire an irregular (cerebriform) nucleus, surrounded by a small halo, and tend to invade the epidermis (epidermotropism), eliciting spongiosis or oedema between keratynocytes. Plaques contain a great amount of atypical, cerebriform, CD3+, CD4+, CD8– lymphocytes, infiltrating the epidermis, the papillary and the reticular dermis (Figure 24.9). Prominent collections of lymphocytes within the epidermis are termed Pautrier microabscesses; these are typical of plaques and rarely found in patches. Mycosis fungoides tumours show a diffuse infiltration of the dermis, with lymphocytes displaying a large variety of cytological appearance, although cells with cerebriform nuclei tend to predominate (Figure 24.10). Epidermotropism in this phase is scarce or totally absent. The CD30 antigen can be expressed in some plaques of

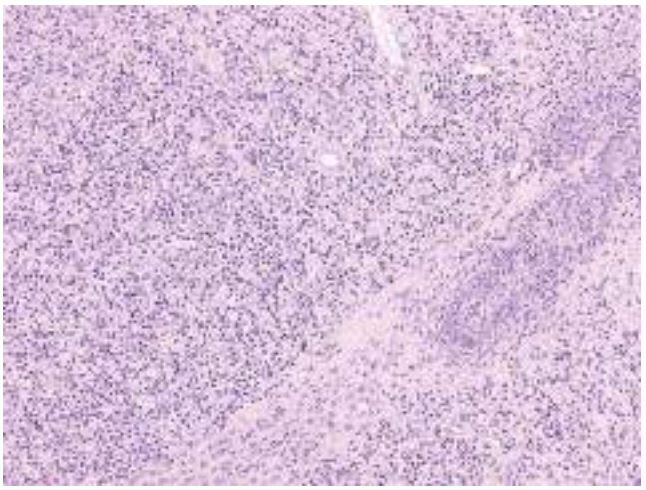


Figure 24.10 Histological appearance of tumours of mycosis fungoides (haematoxylin and eosin stain, magnification 100×).

mycosis fungoides, although it is mostly expressed in tumours harbouring larger amounts of anaplastic cells.

Staging system

Unlike the other B- and T-cell lymphomas, a revised TNM staging system is used for mycosis fungoides (Tables 24.4 and 24.5), which takes into account: the cutaneous involvement (T-stage), in terms of skin surface, presence of tumours or erythema; the involvement of peripheral lymph nodes (N-stage), which should be at least 1.5 cm in their major diameter, along with an evidence of T-cell receptor rearrangement (stages N1b and N2b) or not (stages N1a and N2a) and the involvement of visceral organs (M-stage), which requires pathological confirmation. The blood tumour burden is quantified by the B-parameter, and it reflects the amount of

Table 24.4 Description of TNM-B staging system categories for mycosis fungoides.

Skin	T_1	Limited patches, papules and/or plaques covering <10% of the skin surface. Further, stratify into T_{1a} (patches only) and T_{1b} (plaques ± patches).
	T_2	Patches, papules or plaques covering ≥10% of the skin surface. Further, stratify into T_{2a} (patches only) and T_{2b} (plaques ± patches).
	T_3	One or more tumours, ≥1 cm in diameter.
	T_4	Confluence of erythema, covering ≥80% of body surface area.
Nodes	N_0	No clinically abnormal peripheral lymph nodes: biopsy not required.
	N_1	Clinically abnormal peripheral lymph nodes (firm, irregular, clustered, fixed nodes, ≥1.5 cm in diameter)(*). Further, stratify into N_{1a} (clonally negative) and N_{1b} (clonally positive).
	N_2	Clinically abnormal peripheral lymph nodes(†). Further, stratify into N_{2a} (clonally negative) and N_{2b} (clonally positive).
	N_3	Clinically abnormal peripheral lymph nodes(††). Either clonally positive or negative.
	N_X	Clinically abnormal peripheral lymph nodes; no histological confirmation.
Viscera	M_0	No visceral organ involvement.
	M_1	Visceral involvement (requires histological confirmation; involved organ should be specified).
Blood	B_0	Absence of significant blood involvement: ≤5% of peripheral blood lymphocytes are atypical (Sézary) cells. Further, stratify into B_{0a} (clonally negative) and B_{0b} (clonally positive).
	B_1	Low blood tumour burden: >5% of peripheral blood lymphocytes are atypical (Sézary) cells, but criteria for B_2 are not met. Further, stratify into B_{1a} (clonally negative) and B_{1b} (clonally positive).
	B_2	High blood tumour burden: $\geq 1 \times 10^9$/L Sézary cells clonally positive.

* Histopathology Dutch (lymph node effacement scale) grade 1 or National Cancer Institute (number of atypical lymphocytes within the involved node) LN_{0-2}.
† Histopathology Dutch grade 2 or National Cancer Institute LN_3.
†† Histopathology Dutch grade 3–4 or National Cancer Institute LN_4.

Table 24.5 TNM-B staging system for mycosis fungoides.

	T	N	M	B
I A	1	0	0	0–1
I B	2	0	0	0–1
II A	1–2	1–2	0	0–1
II B	3	0–2	0	0–1
III A	4	0–2	0	0
III B	4	0–2	0	1
IV A_1	1–4	0–2	0	2
IV A_2	1–4	3	0	0–2
IV B	1–4	0–3	1	0–2

circulating Sézary cells (less or equal to 5% versus more than 5%), with at least 1×10^9/L Sézary cells required to diagnose a high tumour burden Sézary syndrome.

Therapy

The treatment of this peculiar kind of cutaneous T-cell lymphoma is specifically stage-adapted (see Table 24.5), which means that different treatment options are applied for each stage of the disease. Patients with an early-stage mycosis fungoides, which is characterised by a slow progression and an indolent course, may take advantage of skin-directed therapies, although lacking a curative intent, whereas more advanced stages require systemic approaches, with either more conventional chemotherapeutic drugs or newer agents.

TNM stage I disease, consisting of patches and/or plaques, requires a skin-directed approach: photochemotherapy, using a psoralen compound as a photosensitising agent and ultraviolet light type A (PUVA), is nowadays the most widely adopted treatment strategy, along with narrow-band ultraviolet light B (UVB) phototherapy (wavelength of 311–313 nm) if patches represent the most relevant cutaneous lesions. Recombinant α_2-interferon may be used with PUVA therapy as a second-line therapy, in order to produce more durable and profound clinical responses; this combination also represents a suitable treatment of stage IIA disease (with patches and/or plaques and lymph node involvement). Unilesional mycosis fungoides may also be treated with conventional external beam radiation therapy.

TNM stage IIB (tumour-stage disease) may be treated either with total skin electron beam irradiation (TSEBI), which yields only 40% satisfactory clinical response, or with systemic

monochemotherapy, usually with gemcitabine or liposomal doxorubicin. The orally bioavailable retinoid Bexarotene is an alternative systemic agent. It is a selective agonist of the retinoid X receptor, which consequently exerts its function through the recruitment of transcription factors, and induces apoptosis and cell cycle arrest in CTCL cells.

Stage III disease (erythroderma) can be first treated again with the combination of recombinant α_2-interferon and PUVA; possible alternatives or second-line options are represented by TSEBI, gemcitabine, liposomal doxorubicin or bexarotene.

Stage IV disease, which is characterised by extracutaneous involvement, requires an immediate systemic chemotherapy: gemcitabine or doxorubicin used as monotherapy should be preferred to polychemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) or CHOP-like regimens, which appear significantly more toxic and display poor disease control.

Histone deacetylase inhibitors (HDACi) are active in patients with advanced-stage mycosis fungoides and in those who relapse or progress. Through the inhibition of the enzymatic removal of acetyl groups from histonic and non-histonic proteins, these molecules control the epigenetic regulation of gene transcription, ultimately regulating cell-cycle and apoptosis-related proteins. Vorinostat and romidepsin, the former being orally available and the latter being administered intravenously, have both shown a rate of response of 30–40% in Phase 2 studies, with a median duration of response varying from nearly 200 days to more than 1 year.

The anti-CD30 immunoconjugate BV demonstrated its activity in CD30+ CTCL within initial phase 2 trials displaying a response rate of 73% and a complete response rate of 35%. The randomised phase 3 ALCANZA trial involving patients with previously treated mycosis fungoides and cutaneous ALCL, BV used as single agent demonstrated superior activity in disease control over standard treatments (oral methotrexate or oral bexarotene). More than half of the patients treated with BV obtained a response lasting at least 4 months compared to roughly 12% of those receiving standard treatments. These results granted BV approval in patients with mycosis fungoides and cutaneous ALCL who have received at least one previous systemic treatment line

Allogeneic transplantation can be a suitable treatment option for younger patients failing at least two previous lines of standard therapy; durable remissions may be obtained as a consequence of a putative graft-versus-lymphoma immune effect, yielding 5-year progression-free and overall survival rates of 34 and 53%, respectively.

Sézary syndrome

Sézary syndrome, first described in 1938 by Sézary and Bouvrain, who identified a population of large lymphocytes in the peripheral blood, with grooved and lobulated 'cerebriform' nuclei (*cellules monstrueuses*), is regarded as the leukaemic form of mycosis fungoides, clinically characterised by the triad: erythroderma (diffuse reddening of the skin), generalised lymphadenopathy and atypical T-lymphocytes (Sézary cells) in peripheral blood, skin and lymph nodes. Like mycosis fungoides, it is a disease of the middle-aged and elderly, although much less common.

Clinical features

Erythroderma is the most relevant clinical aspect; it either tends to evolve gradually over months or even years from a preexisting non-specific dermatitis, or it may abruptly develop *de novo*. Sometimes, erythroderma may reflect an evolution of a patch or plaque stage mycosis fungoides (see above). Skin is red and scaly, and may become doughy, as well as fissurated. Hyperkeratosis of palms and soles is also a distinguishing feature, along with onychodystrophy and sometimes alopecia.

Morphology

Sézary cells are clonal mature T-helper lymphocytes, generally CD3+, CD4+ and CD8–; an aberrant loss of T-cell antigens, such as CD2, CD3, CD4, CD5 and CD7 is frequently observed and T-cell receptor rearrangement found by polymerase chain reaction provides proof of clonality. Histologically, skin changes resemble those of mycosis fungoides, although epidermotropism is seen to a lesser extent. Sometimes, however, skin specimens can be non-diagnostic for mycosis fungoides at all, because there can be more spongiosis and lymphocytes are often small.

Diagnosis

The integration of clinical features, histology, cytology, immunophenotyping and molecular assays is necessary to make a correct diagnosis of Sézary syndrome. In the presence of erythroderma, Sézary syndrome is diagnosed when the absolute count of Sézary cells is $\geq 1 \times 10^9$/L (or Sézary cells represent more than 20% of circulating lymphocytes). Clonal rearrangement of the T-cell receptor is required to establish the diagnosis. Alternatively, one of the following criteria should be satisfied, if a count of Sézary cells cannot be performed: increased CD4+ or CD3+ cells, with a CD4/CD8 ratio $\geq$10, aberrant immunophenotypes (CD4+/CD7– ratio $\geq$40% or CD4+/CD26– ratio $\geq$30%).

Therapy

Extracorporeal photopheresis (ECP) represents the treatment of choice, and it is performed for two consecutive days every 2–4 weeks. During ECP, pooled leucapheresis and plasmapheresis

products are treated with 8-methoxypsoralen, then exposed to ultraviolet light type A (UVA) as they pass through a disposable narrow cassette, *ex vivo*. Irradiated leucocytes are subsequently re-infused: psoralens covalently bind to DNA after irradiation with UVA, therefore leading to the induction of apoptosis. In parallel, ECP seems also to induce dendritic cell differentiation, which in turn translates into an enhanced antigen presentation and the initiation of a host immune response against the tumour. Overall responses to ECP are around 60% and complete responses are seen in about 20% of the cases, with durable responses seen in patients lacking a significant nodal or visceral involvement. ECP can also be combined with TSEBI, α_2-interferon or bexarotene therapy. Systemic chemotherapy based on chlorambucil, methotrexate, fludarabine, gemcitabine or doxorubicin as single agents should be considered in patients with high tumour burden (>2600 Sézary cells/μL), advanced disease with significant nodal or visceral involvement, or relapsed disease.

The humanised anti-CC chemokine receptor 4 (CCR4) monoclonal antibody, mogamulizumab, demonstrated a response rate of 47% in Sezary syndrome patients and 29% in mycosis fungoides within early phase trials, demonstrating the rapid clearance of malignant cells from the bloodstream. In the phase 3 randomised MAVORIC trial where mogamulizumab was compared to vorinostat, superior outcomes in terms of response rate (35 versus 6%) and progression-free survival (8 versus 3 months) were demonstrated in favour of the former. These findings led to the FDA approval of mogamulizumab for mycosis fungoides and Sezary syndrome patients who have failed at least one prior systemic therapy.

Primary cutaneous CD30+ lymphoproliferative disorders

These cover a wide spectrum of indolent T-cell lymphoproliferative disorders, primarily localised in the skin and account for nearly 30% of CTCL, thus representing one of the most frequent disease entities.

They are clinically characterised by a chronic clinical course, with cutaneous lesions sometimes showing a relapsing-remitting behaviour or spontaneous regressions, always featuring the presence of large and atypical CD30+ cells, variably represented within the histological specimen. Primary cutaneous anaplastic large-cell lymphoma (pcALCL) and lymphomatoid papulosis (LyP) represent the two extremities of the spectrum and are discussed in more detail.

Primary cutaneous anaplastic large-cell lymphoma

This is the second most common type of CTCL, being most frequently seen in middle-aged men (median age at presentation is 60 years, with a male/female ratio of 2–3:1). It clinically presents with solitary and localised papules, nodules or tumours, frequently ulcerated, involving the skin of the trunk, face, extremities and buttocks. Multifocal involvement is rather uncommon. Lesions may show a spontaneous complete or partial regression; however, skin relapses are frequent. Extracutaneous dissemination is rare, with regional lymph nodes being the most commonly affected organs other than the skin.

Tumour cells are large, with an anaplastic (abundant cytoplasm, round to oval or irregularly shaped nuclei with prominent nucleoli), pleomorphic or immunoblastic cytomorphology; the infiltrate involves the dermis, without epidermotropism. Cells mostly display a CD4+ T-cell phenotype, CD8+ in a minority of cases, with possible loss of CD2, CD5 or CD3; CD30 is by definition expressed by a majority (>30%) of the neoplastic cells, without CD15 expression. Cytotoxicity markers, such as TIA-1, perforin and granzyme B are frequently expressed.

The prognosis is good, with a 5-year disease-free survival higher than 90%, although relapses are frequent. Patients with multifocal skin lesions and patients with involvement of regional nodes show a similar prognosis to those presenting with a single skin lesion. Localised lesions favourably respond to radiation therapy, and surgical excision may be curative in some cases. Systemic therapy is required for multifocal disease: as demonstrated in the ALCANZA study (see above), BV produces much more significant results than conventional agents like low-dose methotrexate, α_2-interferon or bexarotene.

Lymphomatoid papulosis

LyP is a chronic, recurrent, self-healing skin disease, which mainly affects middle-aged men (with a male/female ratio of 2–3:1). It is clinically characterised by the presence of papular, papulonecrotic or nodular skin lesions, mainly affecting the trunk and the extremities, at different stages of development and with a relapsing-and-remitting course over 3–12 weeks, leaving behind superficial scars. In about 20% of the patients, LyP is associated – or followed by – another malignant lymphoma, such as mycosis fungoides, pcALCL or Hodgkin disease.

LyP has a very heterogeneous appearance upon examination of skin biopsy: for this reason, it has been classified into six distinct subtypes, termed A–E. Type F denotes a folliculotropic variant of LyP not recognised by the current WHO and WHO-EORTC classifications. A newly recognised subtype harbouring the *DUSP22-IRF4* rearrangement has also been described (Table 24.6).

The prognosis is excellent; no treatment is required for localised lesions, while topical steroids, low-dose methotrexate or PUVA phototherapy may be suitable in cases of disseminated disease. BV has an approval for the treatment of LyP, but it is exceedingly rarely required.

Table 24.6 Morphologic and immunophenotypic classification of lymphomatous papulosis, with details on differential diagnoses.

Type A	Most common type, up to 75% of the cases. Wedge-shaped dermal infiltrate of large atypical CD4+/CD30+ lymphocytes within an inflammatory background. Differential diagnosis is with Hodgkin lymphoma, popular mycosis fungoides and transformed mycosis fungoides.
Type B	Small CD4+/CD8-/CD30± lymphocytes with cerebriform nuclei and epidermotropism. Overlaps with mycosis fungoides, clinical correlation necessary to establish the diagnosis.
Type C	Nearly 10% of the cases. Closely resembles type A but it lacks the accompanying inflammatory *pabulum* of neutrophils, eosinophils and histiocytes. Differential diagnosis is with pcALCL, adult T-cell leukaemia/lymphoma, PTCL-NOS and transformed mycosis fungoides.
Type D	Epidermotropic infiltrates of small- to medium-sized pleomorphic lymphocytes expressing cytotoxic markers such as granzyme, perforin and TIA-1. Overlaps with aggressive epidermotropic CD8+ cytotoxic lymphoma, primary cutaneous γ/δ T-cell lymphoma or pagetoid reticulosis.
Type E	Angioinvasive infiltrate of atypical CD30+ lymphocytes in superficial and deep dermis, in a mixed inflammatory background. Differential diagnosis with extranodal NK/T-cell lymphoma nasal type and pcALCL.
Type F	Perifollicular infiltrates of CD30+ atypical cells with folliculotropism, with or without follicular mucinosis. Differential diagnosis with folliculotropic mycosis fungoides and transformed mycosis fungoides.
DUSP22-IRF4	Lymphomatoid papulosis with chromosomal rearrangements of the *DUSP22-IRF4* locus on 6p25.3. Heavy epidermotropism and dense dermal infiltrates of CD3+ lymphocytes.

Conclusions

T-cell lymphoproliferative disorders are classified on the basis of their clinical behaviour and presentation, thus distinguishing them into either indolent or aggressive nodal, extranodal and leukaemic diseases. Although infrequent, they represent a big challenge for the treating haematologists, since their prognosis is rather disappointing when the current treatment strategies are applied, and relapse rates are still relevant. A precise clinical and histological diagnosis, as well as the presence of a multidisciplinary team, are the key elements to assure the best patient management. The application of molecular techniques and immunological markers has clarified some aspects of the pathogenesis of these diseases and has provided the rationale for the development and approval of new specific treatment approaches.

Acknowledgements

The Authors would like to thank Prof. Claudio Agostinelli for providing Figures 24.2, 24.3, 24.4, 24.9 and 24.10.

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CHAPTER 25

Hodgkin lymphoma

25

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Introduction

Hodgkin lymphoma (HL) is an uncommon, relatively aggressive B-cell malignancy with several unique features. Unusually for a cancerous disorder it has a particular predilection for young people, being the most common cancer in the teenage and young adult population. It has a unique pathology characterised by rare, malignant Reed-Sternberg cells within a pronounced inflammatory background. It also has a significant role in the history of cancer treatment: it was one of the first cancers to be cured with the application of linear accelerator-based radiotherapy techniques, followed by similar successes with combination chemotherapy approaches. Often regarded as a success story of modern medicine, HL is one of the more curable malignancies with a sound evidence base of randomised clinical trials guiding modern treatment approaches. Current research focuses on improving outcomes for higher risk groups such as the elderly and those with relapsed disease, and risk-adapting treatment enabling a more personalised approach with de-intensification of therapy where appropriate to reduce late toxic effects.

Pathological features

HL is classified into two broad groups according to the most recent editions of the *World Health Organisation Classification of Tumours of Haematopoietic and Lymphoid Tissues* (2016, 2022); classical HL (cHL) and nodular lymphocyte-predominant HL (NLPHL). Classical HL accounts for the vast majority of cases of HL (95%) compared to NLPHL. Classical HL and NLPHL have distinct pathogenesis, prognosis, clinical features and management. Whilst it may be argued that the WHO classification system which keeps the two entities together is inappropriate (compared with the International Consensus Classification 2022 which has reclassified NLPHL as nodular lymphocyte-predominant B-cell lymphoma), molecular analysis has demonstrated a closer relationship between NLPHL and cHL than between NLPHL and subtypes of non-Hodgkin.

Classical Hodgkin lymphoma

An accurate histological diagnosis of cHL is made by recognising the morphological and immunophenotypic characteristics of the Hodgkin/Reed-Sternberg (HRS) cell within the appropriate cellular background. Systematic immunohistochemical evaluation of both the tumour cell compartment (HRS cells and its variants) and the associated cellular background is important to accurately distinguish cHL from the main competing differential diagnoses; diffuse large B-cell lymphoma (DLBCL) (in particular T-cell/histiocyte-rich diffuse large B-cell lymphoma and primary mediastinal B-cell lymphoma), anaplastic large-cell lymphoma (ALCL) and peripheral T-cell lymphoma not otherwise specified (PTCL-NOS).

Hodgkin and Reed-Sternberg cells

Classic HRS cells are very large (20–50 μm) and can either have one nucleus (termed Hodgkin cells) or be multinucleate (termed Reed-Sternberg cells). HRS nuclei typically contain large, eosinophilic, inclusion-like nucleoli (Figure 25.1). The classic 'owl's eye' appearance describes a binucleate HRS cell with two mirror-image

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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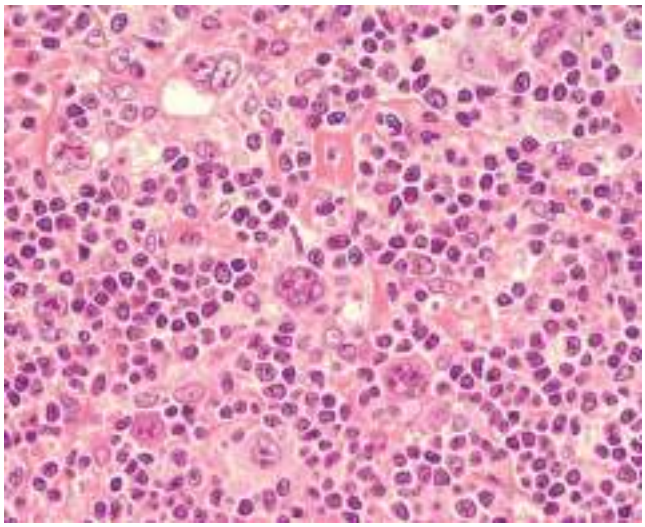

Figure 25.1 Classical Hodgkin lymphoma with numerous multinucleate Hodgkin Reed–Sternberg cells with prominent nucleoli.

nuclei containing eosinophilic nucleoli and a thick nuclear membrane. In most histological subtypes, the HRS cells make up only a small proportion of the cellularity of the tumour (typically 1% or less). High levels of circulating tumour DNA can be detected in most patients with Hodgkin lymphoma at diagnosis and this suggests that there is a high proliferation rate of tumour cells, with a very high rate of apoptosis possibly accounting for the relative scarcity of HRS cells seen histopathologically.

The immunohistochemical hallmark of the HRS cell is positive membrane staining for CD30 (with a paranuclear focus of staining within the Golgi) (Figure 25.2). CD15 is also present in about 70% and useful for the diagnosis. Due to the disruption of the normal B-cell gene expression programme, HRS cells show different immunohistochemical staining characteristics to both normal and malignant B-lymphocytes of other B-lymphoproliferative disorders. Therefore, whilst HRS cells usually stain strongly for MUM1, they show weak (albeit positive) expression of the master B-cell transcription factor PAX5. CD20 may be positive in approximately 20% of cases, but this is usually patchy and weak. CD45 expression is negative in HRS cells; however, due to the surrounding CD45-positive haemopoietic compartment, this feature can sometimes be difficult to appreciate.

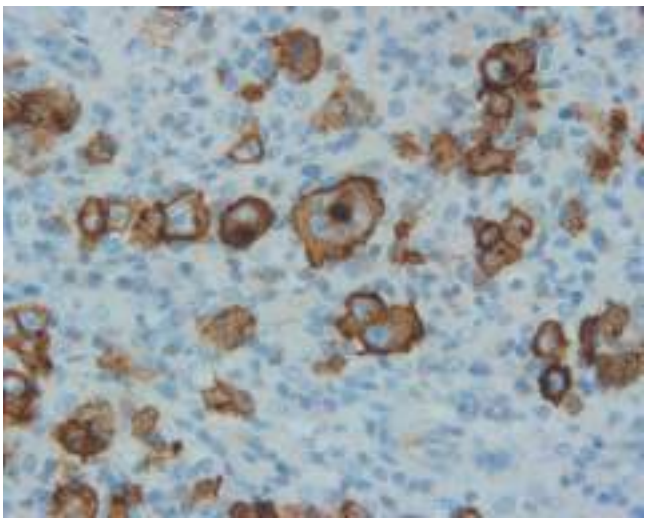

Figure 25.2 CD30 immunohistochemistry of classical Hodgkin lymphoma showing membrane and Golgi apparatus staining of the Hodgkin Reed–Sternberg cells.

Tumour microenvironment

A hallmark feature of cHL is the exuberant tumour microenvironment (TME). The HRS cells have a complex and symbiotic relationship with the TME as evidenced by frequent rosetting of HRS by T-cells of the TME. PD-L1/L2, expressed by HRS cells, interacts with PD-1 expressing T-cells and other immune effector cells such as macrophages, to protect the HRS cells from immunological harm. Numerous cytokines and chemokines are produced by HRS cells, which attract a range of often suppressor-type immune cells including T-lymphocytes (predominantly CD4+ (TH2 and Treg)), macrophages, plasma cells and eosinophils. The cytokines and chemokines produced by HRS cells induce the surrounding inflammatory milieu to produce their own cytokines, leading to propagation of the TME. The immune cells within the microenvironment in turn provide signalling to the HRS cells via surface receptors that activate intracellular pathways, including NF-κB, which contribute to the malignant phenotype of the HRS cells.

Associated histological features

Within cHL, four histological subtypes exist based largely on features of the microenvironment.

- Nodular sclerosis (NS): approx. 70–75% of cases. This is characterised by fibrous bands arising from a thickened capsule that runs throughout the node and compartmentalise the tumour into nodules (Figure 25.3). Lacunar HRS cells are typical of this subtype and are present within a heterogenous cellular background that consists predominantly of CD4+ T-lymphocytes, but also includes histiocytes, eosinophils, plasma cells and neutrophils (Figure 25.4).
- Mixed cellularity (MC): approx. 20–25% of cases. This subtype is named after the heterogenous inflammatory infiltrate that surrounds the HRS cells, consisting of lymphocytes, plasma cells, epithelioid histiocytes and eosinophils. Whilst there may be interstitial fibrosis in the MC subtype, broad bands of compartmentalising fibrosis are not present. The majority of MC cases are EBV-positive.
- Lymphocyte-rich (LR): approx 5% of cases. Compared to the T-cell-predominant lymphocytic infiltrate in NS and MC cHL, this subtype contains a cellular background containing both B

Figure 25.3 Low-power view of lymph node from patient with nodular sclerosis subtype classical Hodgkin lymphoma showing broad bands of fibrosis and thickened capsule.

and T lymphocytes. In addition, there is usually a relative paucity of neutrophils and eosinophils. This form is distinguished from NLPHL by the presence of typical HRS cells, which show membrane staining for CD30 and CD15 (which are not seen in NLPHL).

- Lymphocyte-depleted (LD): <1% of cases. True LD cHL is a very rare entity that histologically may be characterised by either large numbers of HRS cells or rare HRS cells in a densely fibrotic background stroma. The LD histological subtype was reported to be associated with a poorer prognosis; however, many cases were probably misclassified and included cases of ALCL.

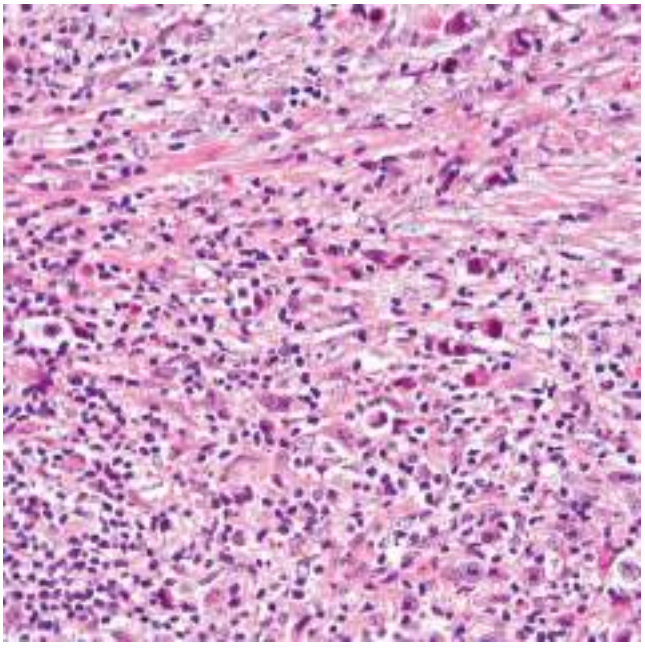

Figure 25.4 Nodular sclerosis classical Hodgkin lymphoma with prominent eosinophils.

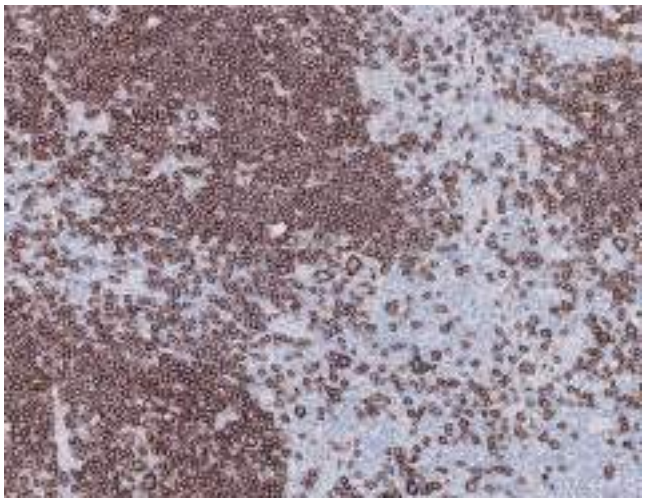

Figure 25.5 Nodular lymphocyte predominant Hodgkin lymphoma stained for CD20 showing nodules of small B cells with scattered larger CD20 positive LP cells.

Nodular lymphocyte-predominant Hodgkin lymphoma

Typically, NLPHL shows a nodular pattern composed largely of small reactive B-cells with infrequent single, scattered LP cells mostly located within the B-cell-rich nodules (Figure 25.5). The lymphocyte-predominant (LP) cells themselves display a fairly typical B-cell phenotype with positivity for CD20, CD79a, PAX5, OCT2 and BOB.1. CD10 is usually negative helping to distinguish them from germinal centre B-cells. CD30 and CD15 are also negative. LP cells can be fairly small, making them hard to spot within the rich B-cell background. However, they typically have a rosette of T-cells which can aid identification. Variant histologies have been described which may differ in terms of clinical presentation and disease course. In general, cases in which the LP cells are found frequently outside of B-cell-rich nodules are thought to be associated with more aggressive disease.

NLPHL can be a challenging diagnosis to make especially when small biopsies are obtained. Wide-bore core, or preferably excision node, biopsies are particularly important for this condition. Progressive Transformation of Germinal Centres (PTGC) is a cause of benign peripheral lymphadenopathy which can uncommonly occur before, during or after a diagnosis of NLPHL. Repeat biopsies are therefore indicated in patients with PTGC who develop newly enlarged lymph nodes. A repeat biopsy is also indicated for patients with NLPHL who relapse after initial therapy or who develop extranodal disease or a rapidly enlarging nodal mass as NLPHL can also transform into T cell/histiocyte-rich large B-cell lymphoma requiring a different treatment approach.

Pathogenesis

Classical Hodgkin lymphoma

Cell of origin

cHL is a B-cell lymphoma although the HRS cell has lost many B-cell associated surface markers such as CD20 and CD79a. This meant the cell of origin for HRS cells was in dispute for many years until clonal rearrangement of immunoglobulin loci was demonstrated within microdissected HRS cells. Somatic hypermutation was also noted within the immunoglobulin loci suggesting transit through the germinal centre. Indeed, some cases appeared to have mutations that would reduce the expression of immunoglobulin, a process that would normally lead to cell death. This led to the concept of HRS cells being crippled germinal centre B-cells which had received an aberrant survival signal preventing normal apoptosis.

Molecular pathogenesis

Genes involved in normal B-lymphocyte growth and differentiation are suppressed in HRS cells. Instead, numerous aberrant intracellular signalling pathways contribute to the malignant phenotype of HRS cells including the following:

- HRS cells show constitutive activation of the NF-κB pathway, which is associated with apoptosis resistance. The molecular basis for this observation includes gains and amplification of the genes encoding the NF-κB factor Rel as well as the kinase MAP3K14 and Bcl3. Mutations in negative regulators also occurs, such as the genes *TNFAIP3* and *NFKBIA*. Other mechanisms can also activate the NF-κB pathway in HRS cells such as signalling through CD40 and the EBV-encoded protein LMP-1 (which can mimic CD40 activation).
- The JAK-STAT pathway is overactive in HRS cells, important in mediating cytokine signalling. Mechanisms of JAK-STAT over-activity include chromosomal gains at 9p24.1-24.3 leading to multiple copies of the *JAK2* gene and inactivating mutations in the negative regulators *SOCS1* and *PTPN1*.
- Numerous other signalling pathways are active in HRS cells. These include the NOTCH pathway, PI3K/AKT pathway and MAPK/ERK pathway. The extent of pathway activation appears greater than for other lymphoma subtypes.
- Immune evasion is a key feature of HRS cells. In addition to harbouring the *JAK2* locus, 9p24.1 contains the *PDL1* and *PDL2* loci resulting in overexpression of the checkpoint proteins PD-L1 and PD-L2 on the surface of HRS cells. This results in protection from killing by PD-1-expressing T-cells and other immune effector cells. Furthermore, frequent inactivating mutations in *B2M* reduce MHC class I expression and therefore recognition by CD8+ T-cells.
- HRS cells have been shown to have deacetylated histones (H3), increased H3K27 trimethylation and DNA methylation patterns, leading to silencing of tumour-suppressor genes and the extinction of the normal B-lymphocyte expression profile.

Role of Epstein-Barr virus

Epstein-Barr virus (EBV) can be detected in HRS cells in a variable proportion of cases of HL, depending on age, sex, race and histological subtype. HRS cells that have destructive mutations of their immunoglobulin loci rearrangements are almost always EBV-positive. This likely represents a mechanism by which the HRS cell bypasses the need for a functional B-cell receptor by using EBV-encoded proteins. EBV infection and expression of latent EBV-associated proteins (LMP1) is also associated with activation of NF-κB as well as JAK/STAT pathways.

Nodular lymphocyte predominant Hodgkin lymphoma

Far less is known about the pathogenesis of NLPHL partly due to the rarity of the disease and difficulty obtaining the rare LP cells from a clinical sample. LP cells harbour clonal rearrangements of immunoglobulin genes and ongoing somatic hypermutation, suggesting a germinal centre B-cell derivation. Similar to HRS cells in cHL, LP cells frequently have an active JAK-STAT signalling pathway which can be due to mutations in *SOCS1*, the negative regulator of JAK2. NF-κB is also often deregulated although pathway mutations are much less commonly seen than in cHL. The microenvironment is composed in most cases of small reactive B-cells although double-positive CD4+ and CD8+ T-cells are seen along with a CD69+ T-cell population, both of which are uncommon in cHL. The rosetting T-cells are typically PD-1+ and PD-L1, expression has been described both on the LP cells and on epithelioid histiocytes in close proximity to LP cells. EBV is not thought to have role in NLPHL pathogenesis although recently IgD-expressing cases have been described to have reactivity to a protein from the bacterium Moraxella catarrhalis.

Clinical features

Classical Hodgkin lymphoma

cHL has an annual incidence of 2–3 per 100,000 in developed countries and has a median age at diagnosis of 35–40 years. It can occur at any age but typically has a bimodal age distribution, with most cases occurring in younger patients but with a second peak in the elderly. cHL usually presents in one of the following ways:

1 Enlarging lymphadenopathy: painless, non-tender, slowly progressive lymphadenopathy in the neck or axillae is a typical presentation. Less commonly, infra-diaphragmatic disease can predominate with palpable inguinal or even pelvic enlarged lymph nodes

2 Complications of lymphadenopathy: compression or invasion of thoracic structures due to a large mediastinal mass is

Table 25.1 Ann Arbor staging system with Cotswolds modifications.

Ann Arbor stage	Criteria
Stage I	Involvement of a single lymph node region (Figure 25.7) or lymphoid structure*
Stage II	Involvement of two or more lymph node regions or lymph node structures on the same side of the diaphragm
Stage III	Involvement of lymph node regions or lymphoid structures on both sides of the diaphragm
Stage IV	Diffuse or disseminated involvement of one or more extralymphatic organs or tissues with or without associated lymph node enlargement. Involvement of liver and bone marrow always considered stage IV
Additional classifiers	
B	Presence of B symptoms defined as • Unexplained weight loss of more than 10% of the body weight during the 6 months before initial staging investigation • Unexplained, persistent or recurrent fever with temperatures above 38 °C during the previous month • Recurrent drenching night sweats during the previous month
E	Limited extranodal extension from adjacent nodal site or apparent discrete single extranodal deposit (excluding liver and bone marrow)
X	A node or nodal mass greater than 10 cm (by largest dimensions of a single node or conglomerate nodal mass). For mediastinal disease, maximum width is equal to or greater than one-third of the internal transverse diameter of the thorax at the level of T5/6 on chest X-ray.

* e.g. spleen, thymus, Waldeyer's ring.

relatively common. Resulting symptoms include chest pain, cough, superior vena cava obstruction (with facial plethora and engorged neck veins) and a chest wall mass due to direct invasion. Less commonly, porta hepatis nodal enlargement can result in biliary tree obstruction leading to jaundice or deranged liver function tests.

3 Systemic symptoms: cHL is a highly inflammatory tumour and is associated with numerous systemic symptoms. Three such symptoms – fever, weight loss and drenching night sweats – are termed 'B' symptoms and have implications for Ann Arbor staging (Table 25.1).

4 *Other symptoms* may occur which are not considered B symptoms. These include fatigue, itch and alcohol-induced pain in affected nodal areas. Other very rare systemic associations include vanishing bile duct syndrome and paraneoplastic cerebellar degeneration.

Nodular lymphocyte predominant Hodgkin lymphoma

NLPHL has a median age at presentation of 35–40 years and shows a male predominance with a 3:1 male: female ratio. Typically, patients present with peripheral lymphadenopathy with no other symptoms and over three-quarters of patients have early-stage disease at diagnosis. The possibility of transformation to high-grade non-Hodgkin lymphoma should be considered in those presenting with B-symptoms, splenic or extra-nodal disease.

Investigations

Classical HL is diagnosed by morphological and immunohistochemical identification of the HRS cell within an appropriate cellular background. The optimal tissue for diagnosis of cHL, and indeed almost all subtypes of lymphoma, is an excisional lymph node biopsy. There is an increasing tendency to perform core biopsies on lymph nodes for the investigation of lymphoma and whilst these are sufficient to make a diagnosis in most cases, there is a risk of a false-negative biopsy due to sampling error. Fine needle aspirates are never acceptable for the diagnosis of cHL and should not be performed if cHL is the suspected diagnosis.

Investigations that are performed in the diagnostic work-up of patients with cHL (and suspected cHL) include:

- Full blood count: typical findings include mild anaemia, leukocytosis and lymphopenia. Eosinophilia and microcytosis are less common associations.
- Renal and liver function tests: to assess baseline organ function, for risk stratification (albumin) and as a screen for hepatic dysfunction due to HL infiltration.
- Erythrocyte sedimentation rate (ESR): for risk stratification in early-stage and paediatric cHL
- HIV serology: the incidence of cHL is increased in patients with HIV
- Echocardiogram/nuclear medicine assessment of cardiac function: to assess for baseline abnormalities that preclude anthracycline treatment

• Respiratory function tests: for patients at significant risk of bleomycin-related lung toxicity or with pre-existing lung disease. The presence of a large mediastinal mass can confound results.

Staging and risk stratification

HL is highly 18-fluorodeoxglucose (FDG) avid, therefore positron emission tomography (PET) imaging is the standard of care for disease staging, usually performed together with CT (Figure 25.6). Approximately 15–20% of patients will be 'up-staged' to a higher disease stage by PET, when compared with CT alone, with implications for treatment selection. PET has high sensitivity for extranodal disease, therefore bone marrow biopsy is no longer routinely required for staging of cHL.

Ann Arbor staging

The Ann Arbor staging system was first developed for cHL in 1971 and remains the principle system for cHL staging for both adult and paediatric cHL today (Table 25.1, Figure 25.7). It is a relevant prognostic factor and is widely used to stratify patients for different treatment approaches. Staging via the Ann Arbor classification involves a thorough history to detect the presence of B-symptoms, examination of affected lymph node groups and radiological investigations to accurately determine the extent of nodal and extranodal involvement. Based on the Ann Arbor stage, patients are usually divided into either early-stage HL (ESHL) or advanced-stage HL (ASHL).

Early-stage Hodgkin lymphoma

ESHL broadly refers to Ann Arbor stage I or II disease. Many groups treat stage IIB as advanced-stage disease, particularly if additional risk factors are present (e.g. extranodal extension and/or mediastinal bulk).

ESHL is often further stratified into 'favourable' and 'unfavourable' or 'intermediate-risk' subgroups according to the presence of specific clinical risk factors, such as the presence of mediastinal bulk extranodal disease, ≥3–4 involved nodal areas, elevated erythrocyte sedimentation rate and/or B symptoms. This distinction has been made in many of the large prospective randomised trials in ESHL to guide treatment selection. Importantly, the criteria used to define favourable and unfavourable are not standardised between international groups, and whilst the criteria are similar, there are important differences (Table 25.2).

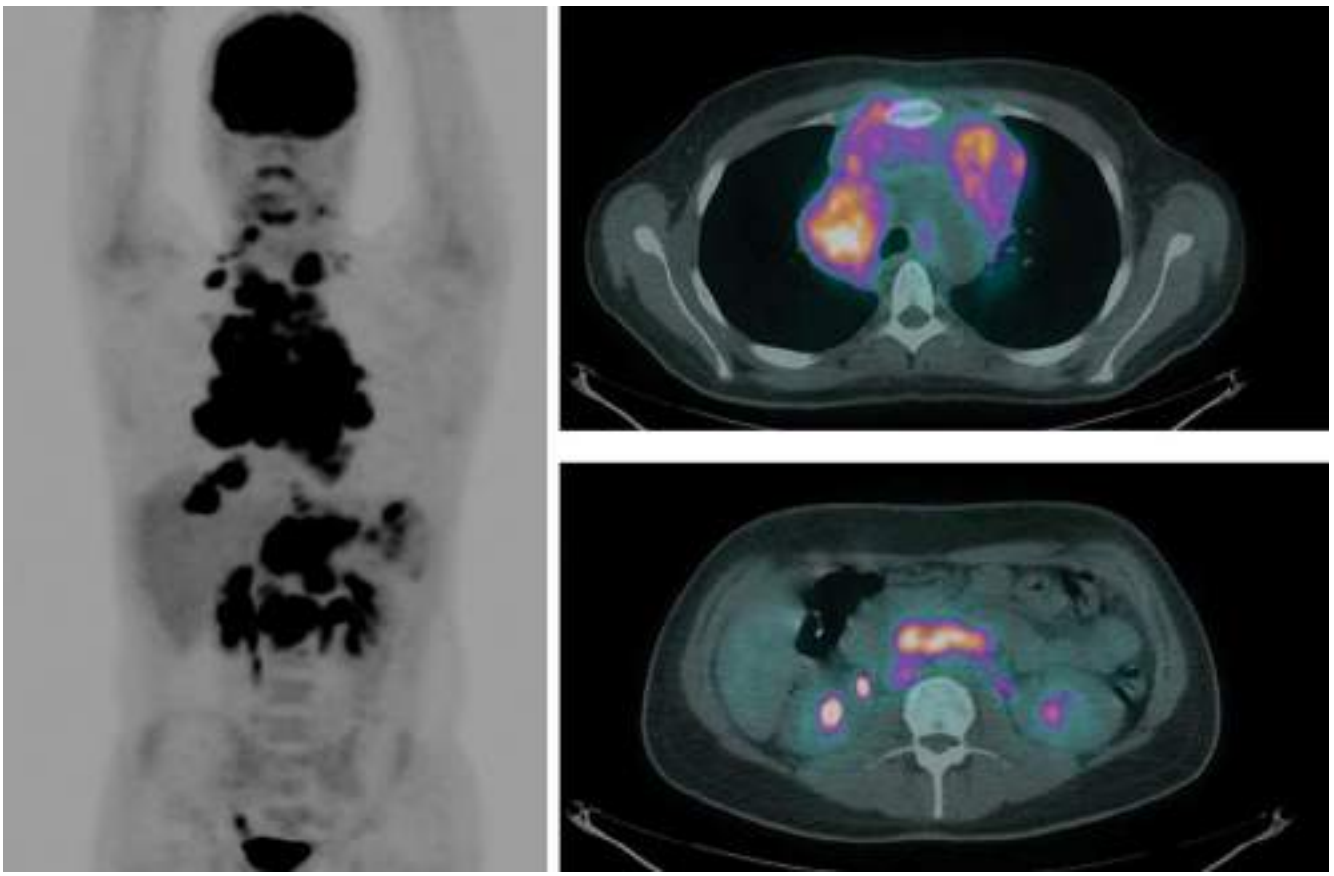

Figure 25.6 PET scan images of patient with stage III Hodgkin lymphoma showing FDG-avid cervical, mediastinal, mesenteric and retroperitoneal lymphadenopathy.

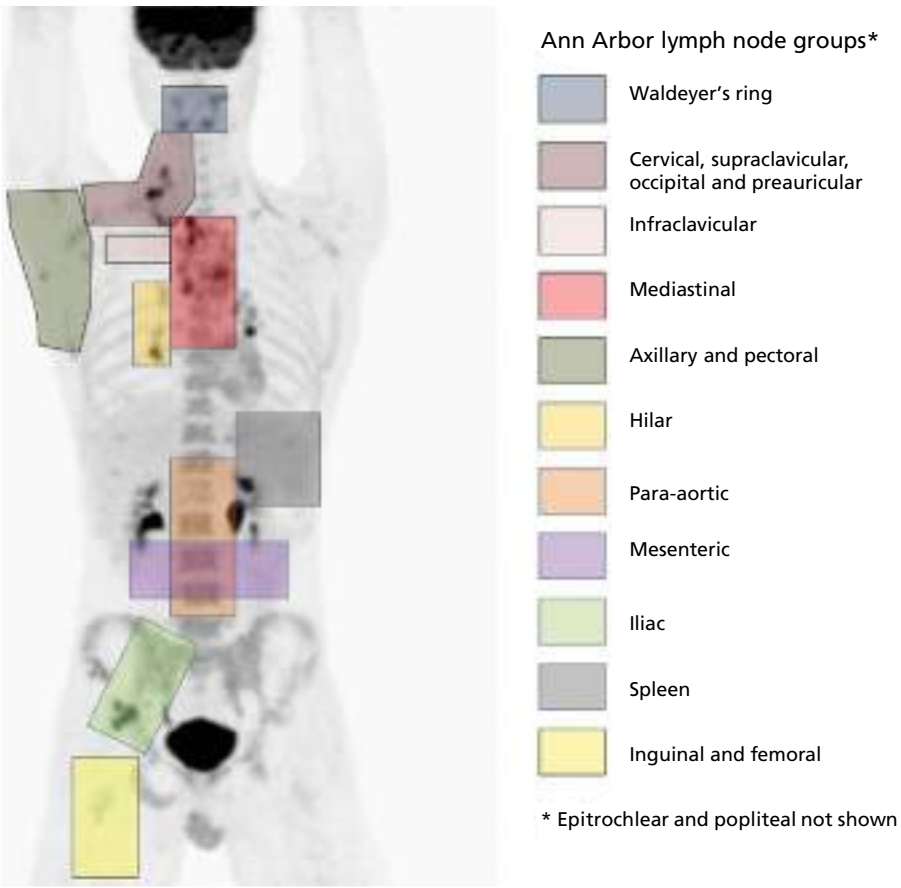

Figure 25.7 Ann Arbor lymph node groups.

Advanced-stage Hodgkin lymphoma

ASHL broadly refers to any patient with Ann Arbor stage III or IV disease. Many prospective trials in ASHL also include patients with stage II disease with high-risk features (e.g. a large mediastinal mass or B symptoms), given that treatment pathways are often shared with ASHL. The International Prognostic Score (IPS), also known as the Hasenclever score, is widely used as a prognostic tool in ASHL (Table 25.3).

Table 25.2 Different definitions of early-stage favourable and unfavourable (or intermediate stage) Hodgkin lymphoma.

EORTC criteria		GHSG criteria	
Early-stage favourable	**Early-stage unfavourable Presence of one or more of the following**	**Early-stage favourable**	**Early-stage unfavourable Presence of one or more of the following**
No large mediastinal adenopathy	Large mediastinal adenopathy	No large mediastinal adenopathy	Large mediastinal adenopathy
ESR <50 without B symptoms	ESR ≥50 without B symptoms	ESR <50 without B symptoms	ESR ≥50 without B symptoms
ESR <30 with B symptoms	ESR ≥30 with B symptoms	ESR <30 with B symptoms	ESR ≥30 with B symptoms
Age ≤50 yrs	Age >50 yrs	No extranodal lesions	Extranodal lesions present
1–3 lymph node sites involved	≥4 lymph node sites involved	1–2 lymph node sites involved	≥3 lymph node sites involved

EORTC, European Organisation for the Research and Treatment of Cancer; GHSG, German Hodgkin's Study Group.

Table 25.3 Outcomes in patients with advanced-stage classical Hodgkin lymphoma using the international prognostic score (patients treated with ABVD or ABVD-like regimens).

International prognostic score	5 yr FFP (%)	5 yr OS (%)
0	88	98
1	84	97
2	80	91
3	74	88
4	67	85
≥5	62	67

Score one point for each of albumin <4 g/dL, haemoglobin <105 g/L, male sex, age ≥45 years, stage IV disease, leucocytosis ($\geq 15 \times 10^9$/L), lymphopenia (<8% of WCC and/or $<0.6 \times 10^9$/L).

Principles of Hodgkin lymphoma treatment

There are three main therapeutic modalities used to treat cHL: conventional chemotherapy, radiotherapy and targeted agents.

Multiagent chemotherapy

cHL is highly chemosensitive and was one of the first malignancies to be cured by multiagent chemotherapy. Combination chemotherapy remains the mainstay of cHL treatment today. Regimens used in frontline treatment usually contain an alkylating agent (e.g. dacarbazine or procarbazine), an anthracycline (usually doxorubicin) and a vinca alkaloid (e.g. vinblastine or vincristine). The most commonly used chemotherapy regimens used for upfront treatment of cHL in adults are ABVD (doxorubicin, bleomycin, vinblastine and darcarbazine), the more intensive escalated BEACOPP regimen (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisolone) and AVD combined with the antibody-drug conjugate brentuximab vedotin (AVD-BV).

Radiotherapy

Radiotherapy is a highly effective modality for the treatment of cHL. Indeed, radiotherapy alone was used as curative therapy for cHL in the 1950–60s. In modern practice, it is predominantly used for localised disease control, particularly for ESHL where it is given as combined modality therapy following multiagent chemotherapy. It also has a role for treatment of refractory disease and palliation.

Older-style radiotherapy fields (e.g. mantle field and subtotal nodal irradiation) typically encompassed widespread nodal areas, as well as normal tissue, resulting in considerable delayed toxicity. Advances in disease staging, together with more effective combination chemotherapy regimens, have enabled a dramatic reduction in field size and radiotherapy dose, therefore modern involved site radiotherapy is far safer. Improvements in radiotherapy techniques, including shielding, deep inspiration breath holding and advances in planning technology, have further refined treatment and reduced exposure to normal tissue. Proton beam therapy is increasingly being used for younger patients with cHL and is predicted to reduce toxicity further for some patients, although long-term data on efficacy compared with standard photon radiotherapy are currently lacking.

Targeted agents

One of the most effective new cHL therapies to emerge is brentuximab vedotin (BV). BV is an antibody-drug conjugate consisting of an anti-CD30 antibody conjugated to the antimicrotubule agent monomethyl auristatin E (MMAE). Ligation of CD30 by BV triggers receptor-mediated endocytosis, leading to lysosomal degradation of BV and release of MMAE within target cells.

PD-1 inhibitors are another very promising class of drugs, with higher single-agent response rates seen in cHL than in solid organ malignancies. This is in part due to the near-universal overexpression of PD-1 ligands on HRS cells, although the mechanism of action is complex and still not completely understood. PD-1 inhibitors are hypothesised to act by stimulating adaptive anti-tumour immunity, although HRS cells frequently downregulate surface major histocompatibility complex (MHC) expression, which hinders effective cytotoxic T-cell responses. PD-1 inhibitors may also block important survival signals between HRS cells and the tumour microenvironment.

Other immunotherapeutic agents in development include CD30-directed chimeric antigen receptor T-cell (CAR-T) therapy, the anti-CD25 antibody-drug conjugate camidanlumab tesirine and bispecific antibodies.

Response assessment, early PET response and response-adapted treatment

PET is the gold standard for response assessment in HL. Residual masses are a common finding after cHL treatment but do not necessarily confer an increased risk of progression if there is no FDG uptake on PET. Therefore, CT alone has limited accuracy for response assessment.

Consensus response assessment criteria for lymphoma are based on PET assessment using the semi-quantitative five-point Deauville scale (Table 25.4). In most situations, a Deauville score of 4–5 is considered 'positive', i.e. uptake greater than normal liver, whilst a Deauville score of 1–3 signifies complete metabolic response (CMR). However, the threshold used to

Table 25.4 Deauville score for the standardised reporting of PET scans in lymphoma.

Deauville score 1	No uptake above background
Deauville score 2	Uptake less than or equal to mediastinal blood pool
Deauville score 3	Uptake greater than mediastinum, but less than or equal to liver
Deauville score 4	Uptake greater than liver
Deauville score 5	Uptake markedly greater than liver and/or new sites of disease
Deauville score X	New areas of uptake unlikely to be lymphoma

define PET-positivity has shifted over recent decades, with many older clinical trials using a more conservative threshold of PET-positivity (e.g. Deauville score 3–5).

Mid-chemotherapy or interim PET response assessment is one of the strongest predictors of outcome in cHL. For the majority of patients (75–90%) who achieve CMR after two cycles of chemotherapy, PFS rates are high (at least 80–95%, depending on treatment and disease stage). However, patients with a positive interim PET scan have inferior outcomes, with PFS rates generally below 50% for ASHL patients receiving ABVD alone. Several trials have investigated interim PET-based response adaptation of treatment with escalation for interim PET-positive patients and de-escalation for PET-negative patients.

Risk-stratified treatment

Similar to response-adapted treatment, risk-stratified treatment attempts to reserve more intensive and toxic therapy from the outset for high-risk patients whilst reducing the intensity of treatment for those with a low-risk

The success of this approach is dependent on accurate assessment of disease risk at diagnosis. Until recently, this has been solely reliant on clinical risk stratification, with different treatment pathways for ESHL patients with 'favourable' and 'unfavourable' baseline characteristics. However, the predictive value of pretreatment clinical risk stratification is suboptimal, particularly in the context of recent advances in PET. In ASHL, some centres recommend more intensive approaches (e.g. eBEACOPP) for patients with a higher baseline risk according to the IPS.

Several promising biomarkers have the potential to improve risk stratification and response adaptation further and hence the precision of recommended treatment pathways. These include advanced PET metrics such as baseline metabolic tumour volume, reflecting overall tumour burden. Circulating tumour DNA and serum cytokines (e.g. CCL17/thymus activation-regulated cytokine (TARC)) may enhance the accuracy of interim response assessment. Further validation is required before incorporating these biomarkers into routine clinical practice.

Frontline treatment

Early-stage favourable cHL

Standard treatment for ESHL without adverse risk factors usually consists of combined modality treatment with ABVD plus radiotherapy. The German Hodgkin Study Group (GHSG) HD10 trial demonstrated that two cycles of ABVD plus 20Gy radiotherapy were equally as effective as four cycles of ABVD plus 30Gy radiotherapy. The subsequent HD13 trial demonstrated that reducing chemotherapy further (i.e. by omitting bleomycin and/or dacarbazine) was associated with inferior disease control. Therefore 2x ABVD plus 20Gy radiotherapy is considered a standard of care.

This combined modality approach is very successful, with overall survival rates >95% at 5–10 years. There is, however, some concern about the late effects of radiotherapy, including secondary malignancy and cardiovascular disease. The NCIC HD.6 trial compared subtotal nodal radiotherapy +/− ABVD with ABVD alone. Radiotherapy was associated with fewer relapses but inferior overall survival at 12 years, largely due to late treatment-related mortality. It is expected that modern involved-site radiotherapy, using lower doses and smaller radiotherapy fields, will markedly reduce the incidence of late toxicity. Nevertheless, these findings instigated a series of trials investigating whether radiotherapy can be omitted altogether for selected patients.

Three key clinical trials have evaluated response-adapted treatment in favourable ESHL. The RAPID, HD16 and H10 trials all randomised patients with PET complete metabolic response (CMR) after ABVD to receive either radiotherapy consolidation or no further treatment (Table 25.5). These trials demonstrated that radiotherapy improves progression-free survival by 4–12%. However, overall survival rates were universally high (≥97%) at 5 years, irrespective of whether radiotherapy consolidation was given. Follow-up remains too short to evaluate the incidence of late treatment-related toxicity, therefore the role of radiotherapy remains a matter of considerable debate. Combined modality therapy remains the standard of care when the risks of radiotherapy are low, however, ABVD alone (three to four cycles) is a reasonable option for patients with higher risk of radiotherapy toxicity who achieve CMR.

Early-stage unfavourable (or intermediate stage) cHL

For patients with ESHL plus risk factors, combined modality therapy has generally been considered standard of care, with 4× ABVD plus 30Gy radiotherapy the most widely applied strategy over recent years.

The GHSG HD14 trial compared four cycles of ABVD with more intensive '2 + 2' chemotherapy, consisting of two cycles of escalated BEACOPP plus 2 ABVD, followed by radiotherapy in

Table 25.5 Outcomes of selected trials in early-stage and advanced-stage classical Hodgkin lymphoma.

Trial	Treatment	Interim PET status	Progression-free survival
Early-stage favourable			
UK NCRI RAPID	ABVD ×3 + RT	CMR (DS 1–2)	94.6% at 3yr
	ABVD ×3		90.8% at 3yr
EORTC/FIL/LYSA H10	ABVD ×3 + RT	CMR (DS 1–2)	99.0% at 5yr
	ABVD ×4		87.1% at 5yr
GHSG HD16	ABVD ×2 + RT	CMR (DS 1–2)	93.4% at 5yr
	ABVD ×2		86.1% at 5yr
Early-stage unfavourable/intermediate risk			
GHSG HD17	BEACOPP(esc) ×2 + ABVD ×2 + RT	CMR (DS 1–2)	97.6% at 5yr
	BEACOPP(esc) ×2 + ABVD ×2		96.0% at 5yr
EORTC/FIL/LYSA H10	ABVD ×4 + RT	CMR (DS 1–2)	92.1% at 5yr
	ABVD ×6		89.6% at 5yr
	ABVD ×2 + BEACOPP(esc) ×2 + RT	PET-positive	90.6% at 5yr
Advanced-stage			
UK NCRI RATHL	ABVD ×2 + AVD ×4	CMR (DS 1–3)	84.4% at 3yr
	ABVD ×2 + BEACOPP ×4	PET-positive	67.5% at 3yr
GHSG HD18	BEACOPP(esc) ×4	CMR (DS 1–2)	92.2% at 5yr
	BEACOPP(esc) ×6–8	PET-positive	89.7% at 5yr
LYSA AHL2011	BEACOPP(esc) ×2 + ABVD	CMR (<1.4× liver uptake)	89.4% at 5yr
	BEACOPP(esc) ×6	PET-positive	70.7% at 5yr
ECHELON-1	ABVD ×6	N/A	76% at 3yr
	AVD+BV ×6	N/A	83.1% at 3yr

BEACOPP(esc), escalated BEACOPP; CMR, complete metabolic response; DS, Deauville score; RT, radiotherapy.

both arms. The more intensive 2 + 2 approach resulted in lower relapse rates but greater toxicity, with no difference in overall survival. The H10 trial evaluated an alternative, PET-adapted strategy, reserving escalated BEACOPP for patients with a suboptimal interim response to ABVD. Patients with a positive PET after two cycles of ABVD were randomised to receive two more cycles of ABVD or two cycles of escalated BEACOPP, followed by radiotherapy in both arms. The latter was associated with superior PFS and a trend towards improved OS.

There is a desire to reduce use of radiotherapy in this group, given that many patients have multiple nodal sites and/or mediastinal bulk and are therefore at greater risk of late toxicity. The GHSG HD17 trial demonstrated that radiotherapy is not required for patients who achieve CMR (Deauville score 1–2) after 2 + 2 chemotherapy. The H10 trial evaluated whether radiotherapy can be safely omitted after ABVD, randomising patients with early CMR to receive either 4× ABVD plus radiotherapy, or 6× ABVD alone. PFS was marginally higher in the radiotherapy arm (2.5% difference at 5 years) and therefore the trial did not demonstrate non-inferiority of a radiotherapy-free strategy. However, given the modest PFS difference, many clinicians feel that it is reasonable to omit radiotherapy after ABVD for patients with CMR where the risks of late toxicity are high. Such patients usually receive response-adapted chemotherapy in line with the advanced-stage RATHL trial.

Treatment selection for 'unfavourable-risk' ESHL patients is complex, balancing cure rates against the acute and delayed toxicities of escalated BEACOPP and radiotherapy, respectively. Response-adapted treatment is now a routine part of care, with interim PET directing treatment intensification after ABVD and/or application of radiotherapy.

Advanced-stage cHL

There are three main approaches for the treatment of adult advanced-stage cHL:

1 Begin treatment with ABVD: Continuing to six cycles remain a standard approach, although many centres will perform a PET scan after two cycles and adapt according to response. The UK

NCRI RATHL trial demonstrated that if patients achieve a negative PET scan at this stage (Deauville score 1–3), omission of bleomycin from the subsequent four cycles results in non-inferior outcomes and reduces toxicity. For patients with a positive PET scan (Deauville score 4 or 5), the trial intensified treatment to a BEACOPP regimen. Although results were better than some historical comparisons using ABVD alone, this arm was not randomised and a 3-year PFS of 67.5% was disappointing. Furthermore, some retrospective data challenges the efficacy of escalation.

2 Begin treatment with escalated BEACOPP: Multiple studies have confirmed escalated BEACOPP to be superior to ABVD in terms of disease control but whether there is an overall survival benefit is less clear. Initial trials of up to eight cycles showed significant toxicity including increased myelotoxicity with more febrile neutropenia and blood product support, and longer-term issues such as a higher risk of infertility and incidence of second malignancy. However, the recent HD18 trial demonstrated that for patients with a negative interim PET scan (defined as Deauville 1–2), four cycles in total were not inferior to six or eight cycles with a significant improvement in short-term toxicity. The impact on late toxic effects is yet to be demonstrated although it is expected to be less. For Deauville score 3–5 after two cycles, patients continued to a total of six cycles. Many centres now treat Deauville 3 as negative with this approach based on outcomes equivalent to the Deauville 1–2 group. Another large, randomised trial called AHL2011, randomised patients with a negative PET scan after two cycles of escalated BEACOPP to continue to six cycles, or to de-intensify to four cycles of ABVD. There was no difference in survival endpoints in the two arms. Although this lengthens the duration of treatment, it reduces toxicity and appears to improve fertility. Whilst this wasn't tested in AHL2011, many centres also omit bleomycin for the four cycles of ABVD.

3 Replace the bleomycin with brentuximab vedotin (BV) and use AVD-BV for six cycles. This is based on the ECHELON-1 trial which randomised over 1300 patients between 6 cycles of ABVD and 6 cycles of AVD-BV. There was no PET response adaptation within this trial. AVD-BV was associated with a 4.9% improvement (corresponding to a 23% risk reuduction) in the 2-year modified PFS. On longer follow-up, a significant overall survival benefit has also emerged with AVD-BV. As expected, there was more neuropathy and febrile neutropenia with AVD-BV necessitating the use of GCSF support, but less lung toxicity. Although licensed in a number of countries, funding remains an issue in some territories due to the cost of BV. Recent trials comparing BrECADD (an intensive brentuximab containing regimen) with escalated BEACOPP and comparing AVD plus nivolumab with AVD-BV have both shown progression free survival benefits and are likely to change frontline treatment approaches in the future.

Radiotherapy in advanced-stage disease

Compared with early-stage disease, radiotherapy has less of an established role in advanced-stage disease. For patients treated with an escalated BEACOPP approach, 30 Gy radiotherapy is given to patients with a residual mass of >2.5 cm which is PET-positive at end of treatment. With this approach, the outcome of patients with an end-of-treatment positive PET was only 6% lower than for the PET-negative group. For patients treated with an initial ABVD approach, the situation is less clear. The RATHL study did not include any protocol-defined radiotherapy and very few patients received it. Whereas initial bulk disease has often been thought to benefit from radiotherapy, a randomised trial of radiotherapy or observation for patients with initial bulk but an interim negative PET scan after two cycles of ABVD showed no benefit for radiotherapy. The trial was small and underpowered however and may mask a clinically significant benefit. Whilst not assessed in a trial, irradiation of sites that are positive on interim or end-of-treatment PET would seem sensible although the risks and benefits of this approach for an individual patient should be discussed with a radiation oncologist.

Paediatric and TYA approaches

Classical HL is the most common malignancy within the teenage and young adult (TYA) age group (13–24 years). Given that the majority of patients are cured with frontline cHL treatment, paediatric regimens have been designed with a strong focus on late effects, fertility preservation and survivorship, aiming to reduce cumulative doses of anthracyclines, bleomycin, alkylating agents and radiotherapy.

The most widely studied treatment in under-18s is the Euronet approach. Patients are stratified into low, intermediate and high-risk groups according to stage, ESR, tumour volume and the presence of extranodal disease. All patients receive two cycles of OEPA (vincristine, etoposide, prednisolone and doxorubicin) followed by one to four cycles of COPDac (cyclophosphamide, vincristine, prednisolone and dacarbazine), dependent on baseline risk group. Radiotherapy is given to slow responders on interim PET, where lower doses are generally employed (19.8Gy) than adult protocols. Progression-free survival rates are 85–90% with this approach.

For older TYA patients (18–24 years), the optimum treatment approach is unknown. Several studies have demonstrated that these patients have comparatively lower progression-free survival rates than paediatric and older adult cHL patients treated with the same regimens. Delays in diagnosis, psychosocial issues and treatment-related side effects may all contribute to this observation.

Treatment of older patients

An estimated 20–30% of patients presenting with cHL in the UK are over the age of 60. Older patients are consistently underrepresented in trials however and there is no defined standard of care in this population. In the German HD10 and HD11 trials in

early-stage disease, only 9% of participants were over the age of 60. Analysis of this subset highlighted that toxicity frequently resulted in failure to deliver protocol-defined therapy and the treatment-related mortality was 5%. Survival was also 10% lower in older patients even with adjustments for deaths not related to cHL or treatment. The risk of bleomycin toxicity is of special concern in older patients with rates of up to 25% reported in some series. The German Hodgkin Study Group do not recommend the use of eBEACOPP in patients 60 years of age or older due to significant morbidity and mortality. A common approach is to use an anthracycline regimen (such as AVD) in fit older patients whereas to use an anthracycline-free regimen (such as modified ChlVPP) in frail patients. Where available, incorporating BV concurrently or sequentially with AVD is an effective approach. Radiotherapy is particularly important in older patients, partly due to relatively poor tolerance of chemotherapy and also due to a low risk of late toxicity, particularly second cancers. Defining optimal treatment in this group is a major aim in cHL clinical research.

Treatment of nodular LP Hodgkin lymphoma

Some countries will enrol NLPHL patients within clinical trials primarily aimed at cHL and draw little distinction between HL treatment approaches. However, it is increasingly clear that the natural history of NLPHL is similar to low-grade non-Hodgkin lymphoma. Long remissions but late relapses are well described, and the prognosis is generally excellent. Treatment approaches are increasingly reflecting this understanding. High-grade transformation to diffuse large B-cell lymphoma (often the T-cell rich subtype) occurs in approximately 10% of patients.

- Early-stage disease: Local treatment approaches are generally applied in this setting such as surgical excision (often as part of the diagnostic process) followed by active surveillance or radiotherapy.
- Advanced disease: For asymptomatic patients, active surveillance is a reasonable approach with an immunochemotherapy combination (such as R-CVP) for those with symptomatic disease. High-grade transformation to diffuse large B-cell lymphoma is a concern for patients with splenic disease, extranodal disease or variant histologies and R-CHOP is often used in this setting.

Relapsed and refractory disease

Treatment of relapsed and refractory (R/R) cHL consists of two or three phases: remission induction, transplant consolidation, and in some instances, maintenance therapy. This approach is potentially curative for at least 50% of patients, if fit and transplant-eligible. Adverse risk factors include extranodal disease, primary refractory cHL or relapse within 12 months of frontline treatment, tumour diameter ≥5 cm and poor performance status.

Remission induction typically involves use of intensive, non-cross-resistant and multi-agent chemotherapy. There are multiple different options, which have never been directly compared in clinical trials, therefore the optimum regimen is unknown. The majority are platinum-based, including GDP (gemcitabine, dexamethasone, cisplatin), ICE (ifosfamide, carboplatin, etoposide) and ESHAP (etoposide, methylprednisolone, cytarabine and cisplatin). Other options include IGEV and BeGEV (ifosfamide or bendamustine plus gemcitabine and vinorelbine). Published response rates vary, but around 50% of patients achieve CMR after two to three cycles of chemotherapy.

Targeted agents are increasingly used in R/R cHL. Brentuximab vedotin (BV) and PD-1 inhibitors (nivolumab and pembrolizumab) are both licensed as monotherapy for R/R cHL, producing high overall response rates, although CMR rates are more modest (typically 20–35%). These are most widely used for patients with insufficient response to initial salvage chemotherapy but are being investigated at earlier stages in the treatment pathway. The KEYNOTE-204 trial demonstrated that PD-1 inhibition with pembrolizumab results in superior PFS and more durable responses compared with BV. The combination of BV or PD-1 inhibitors with salvage chemotherapy as second-line treatment results in high CMR rates (70–90%), but the benefit of these combinations have not been proven within randomised clinical trials.

For fit, non-comorbid patients, the aim is to consolidate with high-dose chemotherapy- most commonly LEAM or BEAM (lomustine or carmustine plus etoposide, cytarabine and melphalan)- followed by autologous stem cell transplant (ASCT). Evidence of chemosensitivity is a prerequisite; PET response to induction chemotherapy is the strongest determinant of outcomes following ASCT. Patients with CMR prior to ASCT have long-term PFS rates of approximately 80%, compared with <50% for patients without CMR. Therefore, ASCT is usually reserved for patients with CMR, or at least very good partial response, to induction chemotherapy. Consolidation radiotherapy is often given together with, or instead of, ASCT for patients with limited-stage disease at relapse or residual PET-positive sites.

An alternative option is allogeneic transplantation (alloSCT) consolidation. There is good evidence for a graft-vs-lymphoma effect in cHL, with low relapse rates following alloSCT, including in patients with suboptimal response (<CMR) prior to transplant. Transplant-related mortality rates have fallen over recent decades, but continue to undermine the feasibility of this approach. AlloSCT is largely reserved for patients who relapse after ASCT or are refractory to multiple lines of therapy with few or no subsequent salvage options.

The AETHERA trial demonstrated that maintenance therapy after ASCT with BV (up to 16 cycles) improves PFS for BV-naïve patients with high-risk R/R cHL (relapse within 1 year and/or extranodal disease). This trial has been criticised for a lack of

mandatory PET assessment prior to ASCT and there is no demonstrated overall survival benefit. Considering the risk of peripheral neuropathy and cost, use of maintenance BV post-ASCT remains contentious. Other maintenance strategies, including PD-1 inhibitor maintenance, are currently under investigation.

Late effects and survivorship

With high cure rates in a relatively young patient population, late treatment-related effects are a major concern for cHL patients and a cause of significant morbidity and excess mortality. The risk of late toxicity depends on patient age, exposure to certain chemotherapy agents, radiotherapy dose and field. The incidence of many late effects, particularly secondary malignancy and cardiovascular disease, increases with time and is often only apparent >10 years after completion of therapy. Therefore, relative mortality rates remain elevated beyond disease cure for most cHL survivors. Indeed, for patients with ESHL and those with ASHL treated with intensive regimens, patients are more likely to die from treatment-related effects than cHL itself.

Secondary malignancies

HL survivors have an approximately fourfold increased risk of developing solid organ malignancy compared to the general population. The most common secondary malignancies are breast, lung and gastrointestinal tract cancers. Radiotherapy increases the risk of secondary solid organ malignancy at exposed sites, with a dose-response relationship. Certain chemotherapy agents are also known to increase the risk of specific malignancies, such as gastrointestinal cancers following procarbazine treatment. Lifestyle factors can modify cancer risk, with a strong association between secondary lung cancers following chest radiotherapy and smoking history.

One of the strongest determinants of secondary malignancy risk is the age at which the patient received treatment. The relative risk of breast cancer risk increases 15–25-fold for patients receiving radiotherapy to breast tissue before age 20, whilst patients aged over 40 have a similar risk to the general population. Sex hormones are a major contributor and these patients should avoid hormonal contraceptives/replacement therapy wherever possible, particularly oestrogen-containing. Breast cancer risk after radiotherapy is lower in patients who have received sterilising chemotherapy with secondary ovarian failure. In the UK, annual breast screening is offered to all lymphoma patients who have received radiotherapy to breast tissue, starting 8 years after treatment or at age 30 years (whichever is later).

Secondary MDS/AML is an uncommon but potentially devastating effect of treatment. It generally occurs 3–10 years after treatment and is related to use of alkylating agents, topoisomerase inhibitors and radiotherapy. It affects approximately 2% patients treated with escalated BEACOPP, compared with <1% with ABVD.

Cardiovascular disease

Cardiomyopathy is a well-established side effect of anthracycline chemotherapy and increases with cumulative dose. Exposure of the heart to radiotherapy confers additional risks of ischaemic and valvular heart disease. As a result, patients treated for cHL have an increased risk of cardiac mortality, depending on patient age and treatment delivered. The risk is greatest in those aged <20 years at diagnosis (up to fourfold increase), and older patients with pre-existing cardiac comorbidities. For paediatric and TYA patients (≤21 years) treated with ≥250 mg/m^2 doxorubicin, the risk of cardiac failure by age 40 is approximately 10%. Concomitant cardiovascular risk factors (e.g. hypertension, hypercholesterolemia, smoking and obesity) compound this risk and should be proactively screened and managed. Surveillance echocardiography is recommended for high-risk paediatric and TYA cHL survivors.

Infertility and premature ovarian failure

The risk of infertility and ovarian failure relates to the cumulative dose of alkylating agents, (e.g. cyclophosphamide and procarbazine) and in rare instances, pelvic radiotherapy. For women of childbearing potential, this is an important consideration in treatment selection. Fertility is preserved in most patients treated with ABVD alone but may be reduced in patients aged ≥35 years. However, 20–30% of ASHL patients will require treatment intensification or further lines of chemotherapy after ABVD, where fertility is often compromised. Women receiving escalated BEACOPP have markedly reduced ovarian reserve and high rates of sustained amenorrhea. This is most evident in women aged ≥30 years, who have <50% chance of regular menstruation following ≥six cycles of escalated BEACOPP, with over a third developing significant menopausal symptoms post-treatment. Fertility preservation should be considered in all young patients with newly-diagnosed cHL, where feasible.

Men are at greater risk of infertility after exposure to alkylating agents than women. There is a ~50% risk of severe oligospermia at 5 years following just two cycles of escalated BEACOPP, rising to >90% after six cycles, whilst the risk of infertility with ABVD alone is generally low (<20%). Pretreatment semen cryopreservation is straightforward for most patients and can mitigate this to an extent. Paediatric trials have shown that substituting procarbazine for dacarbazine markedly reduces the risk of male infertility without significantly compromising efficacy. This substitution is currently

being evaluated in adult regimens, with some centres switching to BEACOPDac rather than BEACOPP.

Other late effects

Osteonecrosis of the hips and/or knees is an uncommon but potentially serious late effect. It is strongly associated with corticosteroid use, age and sex. It is rarely seen after ABVD alone, but occurs in 1–2% patients treated with regimens such as BEACOPP and OEPA/COPDac, increasing to 3% in adolescent males. There is no evidence for any preventative measures, apart from reducing corticosteroid use where possible. Management of established osteonecrosis is supportive.

Conclusion

The high cure rates in cHL are one of the major successes in haemato-oncology. There are now multiple valid treatment options for both ESHL and ASHL. Personalised treatment decisions require multidisciplinary input from both haemato-oncologists and radiation oncologists, taking into account patient preferences and goals. Going forwards, a major priority is to improve the toxicity profile of treatment whilst maintaining high cure rates. To improve the precision of current risk-adapted approaches, more accurate patient risk stratification is required, potentially incorporating novel imaging and circulating biomarkers. There have been major advances in our understanding of the pathogenesis of cHL, particularly with respect to interplay with the immune microenvironment. These have the potential to revolutionise the treatment of cHL over the coming decades, with immunotherapy currently being evaluated in the frontline setting and an ultimate goal of reducing reliance of cytotoxic chemotherapy.

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CHAPTER 26

Indolent non-Hodgkin lymphomas

26

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Introduction

Indolent or low-grade B-cell non-Hodgkin lymphomas (NHL) are a heterogenous group of malignancies with distinct epidemiology, pathogenesis, morphologic, immunophenotypic and clinical features. Despite these differences, they share many similarities in their disease course and treatment paradigms.

Low-grade lymphomas predominantly affect older patients and their collective incidence has increased in line with an ageing population and improved diagnostics. Although still regarded as incurable malignancies, survival rates have dramatically improved with modern treatment approaches including the introduction of anti-CD20 monoclonal antibodies. Improved survival coupled with a rising incidence resulted in a 33% increased prevalence of between 2000 and 2016, reflecting a high burden of disease and a growing need for low toxic therapies to extend the array of treatment options for patients living with low grade lymphomas. In recent years, there have been significant advances in our understanding of lymphoma pathogenesis and many new and promising novel therapies have emerged. Ongoing research is now aimed at deepening our understanding of the biological determinants of risk to delineate patient groups and develop risk-adapted therapies incorporating novel agents to overcome treatment resistance.

This chapter will summarise current management and future directions for follicular lymphoma, marginal zone lymphomas, lymphoplasmacytic lymphoma/Waldenström macroglobulinaemia and mantle cell lymphoma. Chronic lymphocytic leukaemia (CLL)/small lymphocytic lymphoma (SLL) and hairy-cell leukaemia are also mature B-cell malignancies with similarities to the above entities; they are covered in Chapter 23 and T-lymphoproliferative disorders are covered in Chapter 24. Chapter 20 covers the histology and classification of low-grade NHL, but salient features will be highlighted in the relevant sections below.

Follicular lymphoma

Follicular lymphoma (FL) is the second most common B-cell lymphoma accounting for ~20% of non-Hodgkin lymphomas, with an annual standardised incidence of ~3.5/100,000 in Western countries and 0.4–1.1/100,000 in Asia. The median age at diagnosis is 65 years, with a slight male preponderance.

FL typically follows an indolent relapsing and remitting clinical course over many years, with progressively shorter remissions. Therapy resistance or transformation to high-grade disease occurs in 20–30% of patients. A further 20% of patients experience early treatment failure and death; progression of disease within 24 months of the start of first-line immunochemotherapy (POD24) has recently emerged as a powerful predictor of survival with validity across modern immunochemotherapies. POD24 confers a 5-fold increased risk of death at 5 years (50% for POD24 versus 90% for non-POD24), in part attributable to a higher rate of transformation associated with early treatment failure.

Notwithstanding inferior survival for high-risk FL, overall survival has improved considerably in the past 10–15 years with the introduction of therapeutic anti-CD20 monoclonal antibodies and improvements in diagnostics and supportive care. Population-based studies now report median survival exceeding 20 years, challenging the paradigm that FL is an incurable malignancy.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Pathogenesis of FL

FL arises from a common progenitor cell (CPC), typically harbouring the hallmark t(14;18)(q32;q21) chromosomal translocation arising from errors during V(D)J recombination. This leads to constitutive expression of the anti-apoptotic proto-oncogene *BCL2* (B-cell lymphoma-2) and malignant transformation of germinal-centre (GC) B-cells, with effacement of nodal architecture by large, uniform follicles demonstrating various degrees of diffuse neoplastic growth.

Deregulated *BCL2* alone is insufficient for FL to develop. Additional genetic and epigenetic alterations are required. These are typically early clonal events in the CPC pool involving recurrent inactivating mutations of histone modifiers *KMT2D* (*MLL2*) in 79–89% and gain of function *EZH2* mutations in 17–27% of cases. Inactivating mutations of *CREBBP* (32–70%) and *EP300* (9–19%) tend to characterise high-risk FL. Other important epigenetic modifiers in FL include *ARID1A* and *MEF2B*. Mutations affecting NF-κB signalling, B-cell development and cell cycle regulation often occur later. Epigenetic modulation is a fundamental principle in FL pathogenesis and epigenetic-based therapies targeting these early and stable events represent attractive targets for FL and transformed FL.

The CPC undergoes independent and divergent subclonal evolution leading to disease relapse and high-grade transformation, which are additionally linked to upregulation of *MYC*, mutation of *TP53* and other genetic events.

Malignant B-cells exist within a rich tumour microenvironment (TME) composed of various T-cell subsets, macrophages and follicular dendritic cells. The composition and complex interactive 'cross talk' between lymphoma and immune cells are responsible for much of the clinical variability of this disease. Early evidence for the powerful prognostic role of the TME came from a pivotal gene expression profiling study demonstrating superior survival for increased T-cell infiltration (immune response 1 signature) and inferior survival for increased macrophages and dendritic cells (immune response 2 signature). There is considerable interest in further characterising the composition and spatial heterogeneity of the TME to discover predictive patterns associated with treatment resistance and response, with ongoing studies applying highly multiplexed image analysis and other novel approaches of investigation.

Clinical and pathological features of FL

FL commonly presents with painless lymphadenopathy. This is usually asymptomatic but may cause local symptoms or organ compromise. In a large retrospective review, including 4167 patients enrolled in clinical trials, 78% of patients had advanced-stage disease, 19% had B symptoms (weight loss, night sweats or fever), 52% had bone marrow involvement and 38% had extranodal disease outside the bone marrow. CNS involvement is rare in FL, and LDH is elevated in 20% of cases.

FL diagnosis is based on the histomorphologial and immunohistochemical features of FL cells obtained from a needle core or surgical excision biopsy. Malignant cells express B-lineage and Germinal centre (GC) markers (CD10 and BCL6), and most (85%) are BCL2-positive. Bone marrow involvement has a characteristic paratrabecular pattern.

FL GCs contain a monomorphic population of centrocytes without clear zonation. The number of centroblasts per high-power field (HPF) determines histological grade; 0–15 per HPF constitutes grades 1–2 (now collectively termed 'low-grade') and occurs in 75% of cases; >15 per HPF constitutes grade 3, which is further separated into grades 3a (20%) and 3b (5%). Outcomes for grades 1–3a are similar, whereas grade 3b FL has an aggressive course analogous to DLBCL and is clinically managed as such, with the possibility of cure. The proliferation index, as measured by Ki67, usually reflects histological grade and is typically <20% in low-grade FL and >20% in grade 3 disease.

Staging and baseline investigations in FL

Disease staging at diagnosis determines the extent of disease for prognostic assessment and treatment planning and involves imaging of the neck, chest, abdomen and pelvis by CT or PET-CT and a bone marrow aspirate and trephine biopsy. The Lugano 2014 classification system has largely replaced the Ann Arbor system, classifying stage I–II as early or limited stage disease and stage III–IV as advanced stage disease.

FL is uniformly fluorodeoxyglucose (18F) (FDG) avid and FDG-PET is more accurate than CT for staging nodal and extranodal sites, upstaging 18–30% of patients and altering FLIPI score. PET-CT is now the preferred imaging modality for routine staging of FL in updated treatment guidelines, especially for evaluation of early-stage disease to confidently exclude dissemination before recommending involved field radiotherapy (discussed later). FDG-PET is not reliable for assessing bone marrow infiltration in FL, and bone marrow biopsy therefore remains an important component of staging, even when FDG-PET is performed.

Other baseline investigations include full blood count, serum biochemistry, lactate dehydrogenase (LDH), β-2-microglobulin (β2M), immunoglobulins and protein electrophoresis, uric acid and virology for hepatitis B, hepatitis C and HIV. Measurement of creatinine clearance and cardiac function may be required depending on patient age, co-morbidities and therapy choice.

Prognostic markers in FL

Baseline prognostic assessment in newly diagnosed FL is based on clinical and laboratory parameters, with the FLIPI

Table 26.1 Outcomes according to risk group defined by the FLIPI, FLIPI2 and PRIMA-PI scores.

Prognostic tool	**FLIPI**		**FLIPI2**		**PRIMA-PI**	
Adverse risk factors	• Age >60 yrs • Stage III–IV • Hb <120 g/L • LDH >ULN • >4 nodal sites		• Age >60 yrs • BM involvement • Hb <120 g/L • β2M >ULN • Nodes >6 cm		• BM involvement • β2M >3 mg/L	
Risk	**Risk factors**	**5-yrs OS (%)**	**Risk factors**	**3-yrs PFS (%)**	**Risk factors**	**5-yrs PFS (%)**
Low	0–1	91	0–1	91	β2m ≤3, BM neg	74
Intermediate	2	78	2	69	β2m ≤3, BM pos	59
High	3-5	53	3–5	51	β2m >3	39

β2M, Beta-2 microglobulin; BM, bone marrow; Hb, haemoglobin; LDH, lactate dehydrogenase; ULN, upper limit of normal; OS, overall survival; PFS, progression-free survival.

(Follicular Lymphoma International Prognostic Index) and, to a lesser extent, FLIPI-2 scores being the most commonly used (Table 26.1).

The FLIPI score was developed from a retrospective analysis in the pre-rituximab era to predict overall survival (OS). It incorporates age, stage, number of involved nodal groups, haemoglobin level and serum LDH. Three identifiable risk groups had a 10-year OS of 71% for low-risk (0–1 points), 51% for intermediate-risk (2 points) and 36% for high-risk (3–5 points).

A revised system, FLIPI2, was developed prospectively in 2009 to provide a simplified tool to predict progression-free survival (PFS), a more reliable clinical endpoint not confounded by subsequent therapy. The majority (59%) of patients in this study received rituximab-containing regimens, most commonly cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP)/CHOP-like. FLIPI2 determines risk according to age, haemoglobin, presence of bone marrow infiltration, β2-microglobulin, and greatest diameter of largest involved lymph node, the latter providing an easier tool to assess tumour bulk. Three-year PFS was 91% for low-risk (0–1points), 69% for intermediate-risk (2 points) and 51% for high-risk (3–5 points).

Recently, the PRIMA Prognostic Index was developed as an age-independent score based on just two factors – β2-microglobulin and bone marrow infiltration, predicting 5-year PFS rates of 74, 59 and 39% for high-, intermediate- and low-risk FL (see Table 26.1). PRIMA-PI improved risk stratification by overcoming the effect on poor survival of non-relapse mortality in patients aged over 60, identifying a smaller proportion of high-risk patients aged over 60 with greater specificity than FLIPI and FLIPI2. The discriminatory power of PRIMA-PI was shown to be as good as FLIPI.

FLIPI and FLIPI-2 are helpful for describing the distribution of risk in clinical trial populations but their application has not resulted in the development of risk-adapted therapy and they cannot guide choice of therapy. All three tools (FLIPI, FLIPI-2 and PRIMA-PI) were developed and/or validated in a majority of patients treated with CHOP/R-CHOP; their validity across all modern immunochemotherapy regimens is not known.

There is considerable interest in developing baseline prognostic indices to predict POD24 as a validated surrogate for OS. FLIPI and FLIPI2 only predict 50–60% of POD24 events. A novel 11-point model integrating clinical variables and treatment factors from the GALLIUM trial demonstrated superior risk stratification compared to FLIPI and FLIPI2, and improved POD24 sensitivity to 73%, warranting independent validation.

Other promising predictive/prognostic biomarkers in development include total metabolic tumour volume (TMTV) and molecular minimal residual disease (MRD) assessed on circulating tumour cells (CTCs) and cell-free DNA (cfDNA). Models incorporating biological determinants of risk include the m7-FLIPI, which combines FLIPI and performance status with the mutational status of seven genes (*EZH2*, *FOXO1*, *EP300*, *CREBBP*, *CARD11*, *MEF2B* and *ARID1A*), and a 23-gene signature developed from PRIMA trial data reflecting B-cell biology and the TME. Both demonstrated similar sensitivity for POD24 as FLIPI and neither has yet been adopted into routine clinical practice.

Depth of response to induction immunochemotherapy currently provides the most accurate prognostic information for FL patients. A subanalysis of the GALLIUM trial showed that FDG-PET was more predictive of outcome than conventional CT imaging for post-induction response assessment. Using Lugano 2014 response criteria, patients who failed to achieve complete metabolic response (CMR) had a 5-fold increased risk of progression and early death compared to CMR patients (2.5-year progression-free survival 87.4 versus 54.9% and OS 96.6 versus 84.0% for CMR versus non-CMR patients). CMR patients were further stratified by MRD remission status determined by

polymerase chain reaction (PCR) quantification of *BCL2-IGH* rearrangement. Ongoing studies are evaluating the utility of post-induction PET to guide response-adapted therapy to overcome adverse disease biology and are combining models for improved risk prediction; promising results have been reported for combining baseline TMTV plus FLIPI2, TMTV plus end of induction (EOI) PET and MRD plus EOI PET, to mention a few.

Management of early-stage FL

Early-stage disease represents 10–20% of new FL diagnoses. Radiotherapy historically cured ~50% of stage I patients and 25% of stage II FL, including nodal and extranodal presentations staged by CT. Long-term outcomes for this study, involving mostly regional and extended field radiotherapy, were superior for stage I disease (15-year cause-specific survival 87% for stage I versus 54% for stage II) and lymphomas <3 cm in size (15-year rate 49 versus 29% for <3.0 versus ≥3.0 cm). Late relapses beyond 10 years are extremely rare and when they occur, are typically outside the radiotherapy field – perhaps suggesting that occult disseminated disease was present at the time of treatment. More sensitive FDG-PET upstages 23–60% of early-stage cases and, in combination with bone marrow staging, is recommended to confidently identify early-stage cases suitable for radiotherapy; a strategy which has significantly improved long-term disease-free survival compared to historic controls in the CT staging era.

Modern radiotherapy has also evolved to incorporate lower doses and smaller radiation fields, without loss of disease control. One randomised trial, mostly of patients with previously untreated early-stage FL, showed similar outcomes for low-dose involved field radiotherapy (IFRT) (24Gy) and standard doses at the time (40Gy). Very low-dose radiotherapy (4Gy) has also been assessed and, whilst this achieves good disease control and symptomatic relief in the palliative setting, 4Gy is inadequate for long-term disease control.

Despite excellent outcomes for radiotherapy alone, its use is variable. According to a recent National LymphoCare study, over half of early-stage patients in the US received systemic therapy, alone or in combination with radiotherapy. The benefit of systemic therapy in early-stage FL was evaluated in a randomised trial comparing 30Gy IFRT with or without six cycles of cyclophosphamide, vincristine and prednisolone (CVP), with rituximab added latterly for a minority of patients. Combined modality treatment delivered superior PFS and reduced relapses outside the radiation field. There were also fewer transformation events and deaths, however longer follow-up is needed to confirm a significant survival benefit. Nevertheless, results were still excellent for patients treated with IFRT alone (10-year PFS 41%), without the added toxicity of chemotherapy.

A recent retrospective multicentre study produced similar results in patients rigorously staged with FDG-PET and bone marrow biopsy and treated with modern radiotherapy protocols (including IFRT in 50%). Five-year freedom from progression (FFP) and OS rates were 68.9 and 96%, respectively. Five-year FFP was significantly superior for stage I versus stage II disease (74.1 versus 49.1%), confirming that curative potential of radiotherapy for truly localised FL was underestimated in the pre-PET era. The same study demonstrated universally high ORR (96%), PFS and OS rates regardless of treatment modality (radiotherapy alone at doses ≥24Gy, immuno-chemotherapy or combined modality treatment), underscoring the value of radiotherapy alone in these patients.

Taken together, 24Gy IFRT is the recommended approach for early-stage FL, including stage I and contiguous stage II suitable for a single-volume radiotherapy field, and is expected to cure most patients. Chemotherapy or combined modality therapy should be reserved for patients with symptomatic early-stage FL that is unsuitable for single-volume IFRT (including patients with non-contiguous stage II disease) or when radiotherapy increases the risk of serious or long-term toxicity.

Active surveillance should be offered to patients with asymptomatic early-stage disease unsuitable for RT, or when localised disease has been completely excised, the latter being associated with long-term remissions in 80% of cases without additional therapy. Expanding knowledge in the field, and development of biological predictors for progression, may determine a subset of higher-risk patients for whom combined modality therapy may be more beneficial.

Management of asymptomatic low tumour burden advanced-stage FL

Most patients (85%) present with advanced-stage FL. Their need for immediate therapy depends on the presence of high tumour burden features, most commonly evaluated by GELF (Groupe d'Etude des Lymphomes Folliculaires) criteria developed in the pre-rituximab era. The presence of at least one criterion constitutes 'high tumour burden' disease and is sufficient to recommend treatment: a single site of disease >7 cm in maximal diameter, ≥ three nodal sites each ≥3 cm in diameter, systemic or B symptoms, splenomegaly, organ compromise or significant cytopenias (neutrophils $<1.0 \times 10^9$/L and/or platelets $<100 \times 10^9$/L).

Asymptomatic patients with non-bulky disease and no GELF criteria are regarded as having low tumour burden FL. They can be managed with active surveillance as delaying treatment until needed does not compromise disease-specific survival, OS or quality of life. Results of an early, pivotal UK-NCRI trial informed this approach, showing no OS benefit for immediate chlorambucil versus active surveillance in 309 patients. Patients managed with active surveillance delayed systemic therapy for 2–3 years, with ~20% not requiring therapy at 10 years, increasing to 40% for patients aged over 70.

Active surveillance was compared to rituximab monotherapy in a second randomised UK-NCRI study of 379 patients followed for 16 years. Short-course rituximab (weekly doses for 4 weeks) prolonged PFS and doubled the number of patients not requiring therapy within 3 years compared to active surveillance (78 versus 46%), again with no impact on OS. This study provided further evidence that active surveillance does not compromise survival, whilst also proffering short-course rituximab as a reasonable option for patients seeking active therapy to delay immunochemotherapy. Short-course rituximab was subsequently shown to be cost-effective compared to active surveillance, mainly due to the large proportion of patients never requiring systemic therapy.

The value of maintenance rituximab after short-course rituximab induction in low tumour burden FL was evaluated in the RESORT trial. Patients responding to induction rituximab were randomised to maintenance rituximab, 3 monthly until progressive disease, or rituximab re-treatment at relapse, delivered as weekly doses for 4 weeks at each progression until treatment failure. Time to treatment failure (TTTF) was significantly longer for maintenance versus re-treatment (4.8 versus 1.4 years); however, this came at a cost of significant exposure to rituximab (median 18 doses for maintenance versus 4 doses for re-treatment patients) and did not produce a survival benefit. Interestingly, in this trial, 50% of patients randomised to the re-treatment arm remained in remission 3 years after short course induction rituximab. Those that relapsed demonstrated very high response rates to subsequent single-agent rituximab at first and second relapse (61 and 67%, respectively), suggesting that acquired rituximab resistance was not a frequent occurrence in this cohort.

Whether active surveillance is still appropriate in the modern era of novel therapies is an important question that has not yet been addressed; it will be necessary for future trials to determine if novel agents with an acceptable safety profile could change the natural course of this disease to improve survival compared to active surveillance.

Management of symptomatic high tumour burden advanced-stage FL

Patients with advanced-stage FL require therapy when they become symptomatic or meet GELF (or similar) criteria. Modern FL regimens consist of induction immuno-chemotherapy followed by consideration of maintenance therapy in responding patients. Response to first-line therapy, and in particular depth of metabolic response, is the strongest determinant of survival and complete remission produces very long life expectancy exceeding 30 years. Achieving complete metabolic response by post-induction PET has therefore become the most important goal of therapy, alongside symptom control and prolonging time to next treatment.

Induction therapy

Induction therapy has evolved considerably in the past decade, with many studies demonstrating a survival benefit for adding rituximab (R) to first-line chemotherapy. Adding rituximab to CVP (cyclophosphamide, vincristine and prednisolone) significantly improves 4-year OS from 77 to 83%; and adding rituximab to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) significantly improves 2-year OS from 90 to 95%. A large meta-analysis confirmed these findings, concluding a significant improvement in OS for R-chemo compared to various chemotherapy combinations (HR 0.63, 95% CI 0.51–0.79).

Whilst the benefit of adding rituximab to chemotherapy is definitive, the optimal chemotherapy regimen for combination remains unresolved despite evidence from several phase 3 randomised trials. The FOLL05 trial demonstrated inferior TTTF for R-CVP compared to R-CHOP or R-FM (rituximab, fludarabine, mitoxantrone). R-FM was associated with increased toxicity, including neutropenia, infections and second malignancies. This together with a higher rate of myelodysplasia and impaired stem cell mobilisation reported elsewhere led to fludarabine withdrawal from common use in the management of FL. The STiL NHL1 trial showed that bendamustine in combination with rituximab (BR) was more effective and less toxic than R-CHOP (PFS 69.5 versus 31.2 months, HR 0.58, $P < 0.001$), with no difference in OS after 9 years of follow-up. The BRIGHT trial showed that BR was non-inferior to R-CHOP/R-CVP (ORR 97 versus 91%, $p = 0.0102$; CR 31 versus 25%, $p = 0.0225$); OS was not evaluated. Toxicity profiles were different, with RCHOP/RCVP causing more neuropathy and alopecia, and BR caused more vomiting and drug hypersensitivity.

GALLIUM, an recent international randomised phase 3 trial, compared rituximab (R) with the more potent type II anti-CD20 monoclonal antibody obinutuzumab (Gazyvro®, G), both in combination with investigator choice chemotherapy (bendamustine, CHOP or CVP). The study enrolled 1202 previously untreated high tumour burden FL patients. Responding patients (achieving CR/PR to induction therapy) received a maintenance phase of the same antibody for 2 years or until progressive disease. At 34.5 months median follow-up, G-chemo achieved longer PFS than R-chemo (median not reached versus 47.1 months, HR 0.66 (95% CI, 0.51–0.85) $p = 0.0012$) and reduced the POD24 event rate by 46%. This effect was consistent across patient subgroups; however, the trial was not powered to compare different chemotherapy regimens. G-chemo also prolonged time to next treatment (TTNT) and event free survival (EFS: 3-year EFS 87.1 versus 81.2%, HR 0.68, $p = 0.0094$), with a non-significant trend for improved OS. In terms of toxicity, G-chemo caused more grade 3–5 adverse events (76 versus 69%), driven mainly by cytopenias, infections and infusion-related reactions.

CMR was consistently high for both antibodies at a rate >2.5 times higher than CT-assessed CR, but G-chemo increased MRD responses >90%, irrespective of chemotherapy backbone. This was not the case for R-chemo, which produced MRD responses in 76% (R-CVP), 78% (R-CHOP) and 90% (BR) of cases. This trial resulted in the swift approval of obinutuzumab as an alternative to rituximab for first-line treatment of FL in combination with chemotherapy. The observation that obinutuzumab could be combined with less intensive chemotherapy (such as CVP) subsequently led to an anecdotal increase in the use of G-CVP over combinations with other chemotherapies, especially bendamustine, which was associated with significantly more grade 3–4 infections and fatal adverse events than CHOP or CVP, regardless of antibody partner. CHOP in combination with rituximab is generally the preferred choice for clinically suspected or confirmed high-grade transformation, there being no demonstrated benefit of obinutuzumab over rituximab in studies of high-grade NHL.

The novel immunomodulatory agent, lenalidomide, produces high response rates when delivered in synergistic combination with rituximab (R2 regimen). The question of replacing chemotherapy with novel agents was addressed by the RELEVANCE trial, a multicentre, international phase 3 trial designed to demonstrate superiority for R2 over R-chemo (BR, R-CHOP or R-CVP); 1030 previously untreated patients meeting GELF criteria were enrolled, 72% received R-CHOP and 23% received BR in the standard arm. A 2-year maintenance rituximab phase was included in both arms. Both treatments produced remarkably similar outcomes and neither demonstrated superiority for co-primary endpoints (CR at 120 weeks 48 versus 53%, $P = 0.13$; 3-year PFS 77 versus 78%, $P = 0.48$). Grade 3–4 adverse events were also similar with ~30% of patients in both arms not completing therapy, but individual toxicity profiles differed between treatments. R-chemotherapy caused more high-grade neutropenia and febrile neutropenia and R2 caused more high-grade cutaneous reactions. Longer follow-up with more mature survival data are needed to assess long-term outcomes and determine whether R2 should become an option in future for first-line treatment.

Based on current evidence, six to eight doses of either rituximab or obinutuzumab in combination with six cycles of bendamustine, CHOP or CVP is the current standard of induction treatment for previously untreated FL meeting GELF criteria. This is expected to achieve 87–89% ORR by CT assessment and 3-year PFS of 73% (R-chemo) and 80% (G-chemo). In view of the increased risk of infections, patients receiving bendamustine should have extended anti-microbial prophylaxis until recovery of CD4+ T-lymphopenia.

Options for patients unable to tolerate full course immunochemotherapy include attenuated chemotherapy with full-dose rituximab/obinutuzumab, rituximab plus chlorambucil or single-agent rituximab where this is funded. Table 26.2 summarises key trials for frontline induction therapy for symptomatic advanced-stage FL.

Maintenance and consolidation therapy after induction

Up to 2 years of anti-CD20 monoclonal antibody maintenance (with the same antibody used for induction; rituximab or obinutuzumab) provides an option to delay disease progression in patients responding to induction immunochemotherapy.

The landmark PRIMA trial randomised 1019 patients with previously untreated, advanced-stage FL to maintenance rituximab or observation following response to induction R-CHOP, R-CVP or R-FCM. Results demonstrated a clear PFS advantage for maintenance rituximab (375 mg/m^2 every 2 months for 2 years), with updated results reporting a median PFS of 10.5 years for maintenance versus 4.1 years for observation, $P < 0.0001$. Benefits were evident across all FLIPI risk groups and post-induction response categories (PR/CR), but maintenance had no impact on OS, which was high in both arms (10-year OS 80%). These findings were confirmed in a meta-analysis of nine trials of rituximab maintenance involving 2586 patients with FL. The lack of survival benefit suggests that rituximab re-treatment is as effective as maintenance therapy for overall disease control and survival, supported by data from the RESORT trial discussed earlier. Rituximab is now also licensed for subcutaneous delivery, providing a convenient and efficient alternative to intravenous administration, without loss of disease control. This advantage is limited to rituximab as obinutuzumab can only be given intravenously.

Maintenance rituximab improves PFS, but at an increased risk of toxicity which is manageable in most patients but serious in some. Collective results of several trials demonstrate a 5–15% increased risk of neutropenia and delayed post-induction IgG recovery, leading to a >3-fold increased risk of grade 3–4 infections. Considering a lack of a survival benefit for maintenance therapy, clinicians should therefore carefully weigh the benefits and risks of maintenance therapy for individual patients, especially those at increased risk of complications, such as older or co-morbid patients, or those receiving bendamustine-based treatment, where the additional benefit of maintenance has not been assessed in a randomised study.

The advent of more effective induction immunochemotherapy and excellent outcomes for patients achieving CMR has led to investigation of PET-response adapted approaches to determine if maintenance improves disease control in CMR patients. The Italian FOLL12 trial investigated a standard maintenance approach of 2 years' maintenance rituximab given to all CR/PR patients versus a PET-response adapted approach whereby CMR and MRD negative patients omitted maintenance. The results demonstrated a reduction in PFS control when rituximab was omitted but did

Table 26.2 Summary of results of key trials randomised trials of first-line induction therapy for symptomatic advanced stage FL.

First author, year of publication (trial)	Treatment, total number of FL patients	Median follow-up (months)	ORR, CR (%)	PFS (%)	OS (%)
Zucca, 2019 (SAKK 35/10)	R monotherapy, 77	48	57, 36	Median 2.3 yrs	90 at 4 yrs
Marcus, 2008	RCVP, 159	53	81, 41	Median 2.8 yrs	83 at 4 yrs
Hiddemann, 2005 (GLSG-2000	RCHOP, 223	58	96, 44	93 at 1 yr	90 at 2 yrs
Rummel, 2013 (STiL NHL1)	RCHOP, 140 BR, 139	45	91, 40* 93, 76*	Median 2.6 yrs median 5.8 yrs ($P<0.0001$)	Not reached (both arms, ns)
Federico 2013 Luminari, 2018 (FOLL05)	RCVP, 168 RCHOP, 165	84	88, 67 93, 73	52 at 3 yrs 68 at 3 yrs ($p = 0.003$)	95% at 3 yrs (both arms, not compared) 85% at 8 yrs 83% at 8 yrs (ns)
Salles, 2011 Bachy, 2019 (PRIMA)	RCHOP/RCVP, 491 (no R maintenance) RCHOP/RCVP, 499 (with R maintenance)	118	99, 70	Median 4.1y Median 10.1y (P<0.001)	>90 at 3y 80 at 10y (both arms, ns)
Marcus, 2018, GALLIUM	RCVP/RCHOP/BR, 601 GCVP/OCHOP/OR, 601 (both arms with R/G maintenance)	35	87, 24 89, 20	73 at 3 yrs 80 at 3 yrs ($P = 0.001$	92 at 3 yrs 94 at 3yrs (ns)
Fowler, 2017 RELEVANCE:	RCVP/RCHOP/BR, 517 R-lenalidomide, 513 (both arms with R maintenance)	38	68, 53 65, 48	78 at 3 yrs 77 at 3 yrs (ns)	94 at 3 yrs 94 at 3 yrs (ns)

BR, bendamustine, rituximab; G, Gazyvro®, obinutuzumab; ns, not statistically significant; OS, overall survival; PFS, progression-free survival; R, rituximab; RCVP, rituximab, cyclophosphamide, vincristine, prednisolone; RCHOP, rituximab, doxorubicin, cyclophosphamide, vincristine, prednisolone; y, years.
* Showing response rates for FL, MCL, MZL and WM.

not directly evaluate the effect of maintenance versus no maintenance in CMR patients. This question, along with investigating the role of novel agents to improve outcomes in <CMR patients with the addition of lenalidomide to maintenance schedules, is being addressed in the ongoing UK-NCRI PETReA trial.

The optimal duration of maintenance is not known. Results from low-risk/low tumour burden studies suggest no benefit for extended duration maintenance (up to 5 years) or for prolonged maintenance over re-treatment at relapse, and it seems reasonable to extrapolate these data to high tumour burden FL; accordingly, it is standard practice for maintenance therapy to be delivered for 2 years.

Radio-immunotherapy consolidation with ^{90}yttrium-ibritumomab tiuxetan or ^{131}iodine-tositumomab has been an option to improve CR and PFS in patients responding to induction. Although effective and generally well tolerated, an increased risk of myelodysplasia has been observed and this, coupled with specialist delivery requirements, led to declining use largely superseded by maintenance antiCD20 monoclonal therapy.

Autologous stem cell transplantation (ASCT) consolidation in first remission is not recommended for FL patients based on results of four randomised trials comparing ASCT to observation, mostly performed in the pre-rituximab era. All but one of these trials demonstrated significant improvements in PFS, but this did not confer a survival benefit, partly due to excess toxicity (including secondary malignancies) in the ASCT arms and the success of salvage therapy in patients not receiving ASCT. In the current era of highly efficacious induction therapy and maintenance therapy, which has a low toxicity profile, there is no role for more toxic ASCT consolidation in first remission; ASCT does however remain an important therapeutic option at relapse (discussed later).

Management of relapsed FL

Most patients with advanced-stage FL will experience one or more relapses of disease. A biopsy should be performed to exclude high-grade transformation, especially in cases of early treatment failure after highly effective first-line immunochemotherapy with bendamustine or CHOP, or when transformation is clinically suspected. A recent competing risk analysis demonstrated a 23% risk of transformation for POD24 patients compared to just 3.4% for patients without POD24; risks increased to 42% for patients progressing within 12 months. Suspicions for transformation should not be based on FDG-PET uptake alone, as many FL cases have increased FDG uptake without a high-grade component. Management of transformed FL is discussed below.

There are many options but no standard treatments for relapsed FL. Management of localised or asymptomatic advanced-stage relapse without GELF criteria is the same as the first-line setting. For localised recurrence, 24 Gy is highly effective but lower doses (4 Gy) may be appropriate, especially for symptom control. Active surveillance is appropriate for asymptomatic, advanced-stage disease without GELF criteria. Patients with symptomatic advanced-stage, non-transformed relapse should be considered for immunochemotherapy or novel therapy when GELF criteria are met. Re-treatment with single-agent rituximab is also an option, especially for low tumour burden disease or elderly patients, resulting in a median TTTF of 4 years; however, rituximab monotherapy is not universally reimbursed in this setting, with most clinicians offering immunochemotherapy when patients become symptomatic, with dose reductions if patients are frail or elderly. There are no evidence-based guidelines for optimal therapy sequencing, thus choice of therapy is an individualised decision based on previous treatment received, associated toxicity, quality of response and duration of remission. Patient age, co-morbidities and preference, as well as the availability of options, are central to these decisions.

Non-cross-resistant immunochemotherapy is the mainstay of treatment for relapsed FL. Re-treatment with R-bendamustine or R-CVP may be considered if a prolonged remission (>2 years) was previously achieved, but there is limited scope to repeat anthracycline-based treatment such as R-CHOP due to an excess risk of cardiotoxicity. Rituximab plus lenalidomide (R2 regimen) was recently licensed for relapsed/refractory FL from the second treatment line onwards based on results of the AUGMENT and ongoing MAGNIFY trials demonstrating a PFS advantage over rituximab monotherapy and a median PFS of 39–40 months. In the first relapse setting, expected response rates for treatment are 85% for R-CHOP, 82% for BR and 78% for R2.

The positioning of R2 in the treatment pathway for relapsed/refractory FL has not been established as there have been no direct comparisons with immunochemotherapy at relapse. The option is attractive to both older and younger patients, with equivalent efficacy and safety outcomes reported for patients aged over and under 70 years, and provides a rational non-chemotherapy option for patients with early treatment failure, most of whom will be chemo-refractory. Median PFS for the POD24 subset treated with R2 in the AUGMENT trial was 30.4 versus 39.4 months for non-POD24 patients. R2 is also suitable for rituximab refractory patients, as it synergises with rituximab for enhanced antibody-dependent cell-mediated cytotoxicity to overcome resistance.

When previous remissions lasted less than 2 years, an alternative, non-cross-resistant immunochemotherapy or novel therapy is preferable; the main choice of chemotherapy backbone for immunochemotherapy is CHOP or bendamustine. Some centres may use a platinum-containing regimen for transplant candidates (discussed later) who have previously received CHOP-based therapy, or progressed on standard regimens. Obinutuzumab in combination with bendamustine, followed by obinutuzumab maintenance, is an option for patients with rituximab-refractory disease, defined as resistance or relapse within 6 months of any rituximab-based regimen. This offers a median PFS of 29.2 months compared to 14 months for bendamustine monotherapy according to results of the GADOLIN trial, which also demonstrated an OS benefit compared to bendamustine monotherapy.

Several randomised trials and a meta-analysis have demonstrated that rituximab maintenance leads to significant improvements in both PFS and OS over observation alone after second-line therapy, including patients who have previously received rituximab-containing immunochemotherapy. The largest trial reported an improvement in median PFS from 14.9 to 51.5 months, but it should be noted that these patients had not received maintenance after first-line therapy. In practice, a second round of maintenance is seldom applied, mainly due to risks of cumulative toxicity with prolonged B-cell depletion. Maintenance after re-induction at relapse is generally reserved for patients with high-risk disease who have not previously received maintenance therapy.

Several PI3-kinase inhibitors were FDA/EMA approved for relapsed and refractory FL, including idelalisib, copanlisib, duvelisib and umbralisib. These agents, which target the deregulated PI3K pathway important for B-cell receptor signalling, cellular proliferation and anti-tumour immunity, typically produce ORR rates of 40–60%, but CR rates are low (4–20%) and median duration of response is ~10–14 months in heavily pre-treated patients (median of 3–4 prior lines of therapy). The major limitation of these agents is their safety profile, with an increased risk of severe infections and autoimmune side effects, especially with continuous and long-term dosing, mandating the use of antimicrobial prophylaxis and monitoring for infections. Intermittent dosing schedules are under investigation to improve safety and preserve efficacy, and next-generation agents with improved specificity, such as umbralisib, appear to have improved toxicity profiles. Access to these agents varies considerably; none are

currently available in the UK and most licenses have been withdrawn due to unacceptable safety profiles.

Tazemetostat, an oral inhibitor of the enhancer of zeste homologue 2 (EZH2), was recently FDA-approved for relapsed/refractory FL harbouring *EZH2* mutations or from the third treatment line onwards without available standard options. Results from a phase 2 trial demonstrated higher response rates for *EZH2*-mutant FL compared to wild type (ORR 69 versus 35%) but PFS rates were similar (median PFS 13.8 versus 11 months ($EZH2^{MUT}$ versus $EZHT^{WT}$).

Radioimmunotherapy (RIT) is an effective alternative to immunochemotherapy for relapsed FL, but there are no direct comparisons of RIT in the relapsed setting against other modalities. Zevalin (^{90} Yttrium-ibritumomab tiuxetan) produces high response rates with durable remissions in heavily pretreated (albeit rituximab-naïve) patients, but restricted access to (few) specialist centres and the availability of novel treatment alternatives has largely replaced this agent.

All patients with relapsed/refractory FL should be considered for experimental therapies if clinical trials are available. Emerging novel therapies for the treatment of FL are discussed below.

Transplantation in relapsed FL

Both autologous and allogeneic haematopoetic stem cell transplant (HSCT) have a role in a subset of patients with relapsed or transformed FL.

Several non-randomised studies from the pre-rituximab era suggested that ASCT prolongs PFS in relapsed FL compared to historical controls treated with standard chemotherapy. Results of the European CUP trial also showed a survival benefit for ASCT after CHOP re-induction (2-year OS, 71 versus 46%, HR 0.4, $P = 0.026$). Several studies indicate a plateau in the survival curves approximately 10 years after ASCT, with 12-year OS of 48% in one study suggesting that some patients may be cured.

Recent retrospective observational studies highlight an emerging role for ASCT in FL patients with early treatment failure after first-line immunochemotherapy. One study showed a 20–25% improvement in 5-year survival for ASCT compared to observation in POD24 patients treated mostly with immunochemotherapy. Another study identified a survival benefit only for patients undergoing ASCT within 1 year of early treatment failure. Although retrospective and subject to selection bias, these studies highlight the potential value of ASCT as an early management strategy for high-risk relapsed FL. Myelodysplastic syndrome (MDS), acute myeloid leukaemia (AML) and other second malignancies account for the majority of late non-relapse mortality after ASCT and must be considered when weighing options, especially with increasing availability of alternative novel options.

Allogeneic HSCT offers the possibility of cure for a subset of younger patients with high-risk relapsed FL and a suitable donor. Reduced-intensity conditioning (RIC) HSCT is preferable to myeolablative conditioning for FL, due to its lower transplant-related mortality (TRM) and enhanced graft-versus-tumour effect. Several retrospective and registry studies of RIC allogeneic HSCT in FL suggest a relapse rate of 10–44% and TRM of 11–40% at 3 years, delivering 3-year OS rates of 50–65%. At present RIC allogeneic HSCT is recommended for patients who relapse after prior ASCT. While it is tempting to consider alloSCT in younger patients with early relapse and high-risk disease characteristics, there have been no prospective trials assessing autologous versus allogeneic transplantation and the advent of highly effective novel therapies may soon challenge the role of alloSCT in this setting.

Novel therapies for FL

A burgeoning array of novel agents is in clinical development for FL, with a strong emphasis on improving outcomes for high risk and multiply relapsed FL. Some are already available for routine use, including lenalidomide, rituximab and tazemetostat (discussed earlier).

Bispecific monoclonal antibodies simultaneously targeting CD20 and CD3, and CD-19 directed chimeric antigen receptor T-cell (CAR-T) therapies are amongst the most promising emerging agents. Impressive ORR and CR rates have been reported in early phase trials of high risk and heavily pre-treated patients, but long-term efficacy and safety are pending mature follow-up data. The four leading bispecific antibodies (mosunetuzumab, glofitamab, odronextamab and epcoritamab) demonstrate ORR of 62–92% and there are other similar molecules in development too. Most patients achieve CR (50–60%), and for these patients, responses appear to be durable. Recent data for CAR-T therapies are also very encouraging; axicabtagene ciloleucel (ORR 94%, CR 80%, median PFS not reached after 17.5 months of median follow-up) and tisagenlecleucel (ORR 83%, CR 65%, median PFS not reached after 9.9 months median follow-up). Predictable toxicities of these immune-activating therapies include cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome (ICANS), mostly low-grade in FL, and cytopenic complications necessitating specialist delivery units to manage these patients.

Other novel agents with high activity in FL include the antibody-drug conjugates loncastuximab tesirine (CD19-directed), polatuzumab vedotin (CD79B-directed), the radioimmunoconjugate lutetium (177Lu) lilotomab satetraxetan (CD37-directed).

Since the success of the R2 combination, there has been considerable interest in combining lenalidomide with other novel agents, including the bispecific antibodies, obinutuzumab and novel antibody-drug conjugates. The combination of obinutuzumab and lenalidomide (GALEN regimen) produces durable remissions, with 2-year PFS exceeding 65%.

Table 26.3 Suggested algorithm for management of FL.

- Grade 3b and transformed FL: treat as high-grade lymphoma.
- Early-stage disease confirmed by comprehensive staging (bone marrow aspirate and trephine and FDG-PET scan, if available): 24 Gy involved field radiotherapy; other options should only be considered within clinical trials.
- Advanced-stage asymptomatic low tumour burden disease: active surveillance or short course rituximab monotherapy, with choice depending on patient wishes and goals of treatment.
- Symptomatic advanced-stage disease: six to eight cycles of rituximab or obinutuzumab in combination with six cycles of chemotherapy. Obinutuzumab-chemo prolongs PFS compared to rituximab-chemo. The chemotherapy options are bendamustine, CHOP or CVP; Bendamustine and CHOP, are superior to CVP. Fludarabine-based treatments are no longer recommended.
- Patients who are unable to receive full-dose immunochemotherapy: attenuated chemotherapy with full-dose rituximab/obinutuzumab or rituximab alone.
- Consolidation of first remission with rituximab/obinutuzumab maintenance for 2 years is recommended in most instances, with evidence that this prolongs remissions and time to next treatment, but no evidence that it prolongs survival.
- Relapsing patients should be re-staged and repeat biopsy performed to exclude high-grade transformation. Treatment depends on stage at relapse, prior treatments and duration of previous remission and is dictated by the availability. Options include R-chemotherapy, obinutuzumab-bendamustine, Rituximab-lenalidomide. Tazemetostat and several Pi3 kinases are licensed but availability is variable.
- ASCT is a useful treatment option for patients achieving second or subsequent remissions and should be considered early in the disease course and for high-risk patients.
- Allogeneic haematopoietic stem cell transplantation after reduced-intensity conditioning can be considered in younger patients, with a suitable donor, who have aggressive early relapsed disease, especially after prior ASCT.
- Transformation has an inferior prognosis and optimal treatment is unknown. Options include RCHOP and salvage regimens (e.g. RGDP) with ASCT in patients achieving remission, except in treatment naïve patients presenting with high-grade transformation at first diagnosis.
- Novel therapies are highly promising, and good-quality randomised trials are required to understand how these treatment options should best be used. Patients should be enrolled on a clinical trial wherever possible.

BTK, BCL2 and PD1/PDL1 checkpoint inhibitors, which have broad clinical activity and application across a wide range of lymphomas, have generally yielded disappointing results in FL when delivered as single agents. Some combination strategies are still under investigation, and biological insights are emerging.

With such a large array of promising emerging therapies, it is likely that the treatment landscape for FL will undergo significant transformation in the coming years. The major challenge will be to determine the role and place of novel agents in modern treatment pathways and to develop surrogate endpoints and novel predictive and prognostic biomarkers for personalised approaches and optimal patient selection. Table 26.3 provides a suggested algorithm for management of FL.

Management of transformed FL

Transformation to high-grade lymphoma, most commonly to DLBCL, but occasionally Burkitt lymphoma, occurs at a rate of ~3% per year or 15–28% at 10 years. There is considerable variation in reported rates of transformation, in part due to differences in the definition of transformation (whether histologically confirmed or clinically suspected). The large co-operative ARISTOTLE study, involving 11 groups and 10,000 patients, recently reported a 10-year cumulative incidence of 7.7% for high-grade transformation defined as a biopsy-proven first event after first-line therapy. In this study, the addition of rituximab significantly reduced the risk of transformation; however, prior rituximab treatment had no impact on survival rates after transformation, which was reported to be 32% at 10 years – an improvement compared to historic controls.

High-grade transformation often occurs relatively early in the disease and may be accompanied by B symptoms, high LDH and nodal or extranodal masses growing rapidly and often discordantly from other involved areas.

There is limited evidence to direct optimal therapy for transformed FL. CHOP should be offered to patients who have not previously received anthracyclines, alternatively, platinum-based salvage regimens may be preferred, usually in combination with rituximab for CD20-positive tumours. Radiotherapy consolidation after immunochemotherapy is an option for limited-stage disease. Consolidation with high-dose therapy followed by ASCT is often performed for transplant-eligible patients undergoing high-grade transformation after previous treatment for FL; however, previously untreated patients with transformed FL at the time of initial diagnosis have a good outcome following R-CHOP (analogous to *de novo* DLBCL) and ASCT consolidation in first remission may be avoided in these cases.

Marginal zone lymphomas

Marginal zone lymphomas (MZLs) arise from chronically stimulated immune cells within expanded marginal zones of secondary lymphoid follicles. Pathogenesis is linked to genetic factors, autoimmune conditions and infections leading to chronic antigenic stimulation and inflammation.

MZL accounts for 7–8% of mature B-cell neoplasms. The incidence is increasing over time and rises sharply with age for all subtypes; in the UK, the median age at diagnosis is 72 years and although individual subtypes are rare, MZL may collectively now be more common than FL. There are three genetically, clinically and therapeutically distinct histological subtypes – extranodal lymphomas of mucosa-associated lymphoid tissue (MALT; 61%), nodal MZL (NMZL; 30%) and splenic MZL (SMZL; 9%). All are characterised by the presence of mature B-cells expressing surface immunoglobulin, CD20 and pan-B-cell markers, typically negative for CD5 and CD10, and with variable CD23 expression. Histological subclassification and differential diagnosis from other indolent B-cell lymphomas may be difficult in cases presenting with atypical (usually CD5 positive) histology or disseminated disease involving nodal and extranodal sites, peripheral blood, bone marrow and spleen.

The best staging system for MZL is controversial as neither the Ann Arbor system nor the 2014 Lugano classification is ideal. CT is routinely used for staging and response assessment, in addition to site-specific investigations for MALT lymphomas. The use of FDG-PET in MZL is an open question due to heterogeneity of FDG uptake. Recent data indicate that most MZL lesions are detectable by PET, especially nodal subtypes, and PET may upstage a proportion of patients compared to CT; however, the impact of PET staging on treatment decisions and the prognostic value of PET for treatment response remain investigational. At this time, PET in MZL is generally reserved for cases with suspected high-grade transformation.

First-line systemic therapy for MZL

The management of MZL follows similar general principles to other low-grade B-NHLs, with additional roles for anti-infective therapy and surgery in selected subtypes. Localised disease (stage l and contiguous stage ll) can be treated with potentially curative radiotherapy; advanced-stage asymptomatic disease can be observed, without compromising OS; and symptomatic advanced-stage disease, at initial presentation or relapse after antibiotics or local therapy, is typically treated with rituximab or rituximab-containing immuno-chemotherapy, with novel targeted therapies beginning to emerge. The choice of chemotherapy depends on patient age, fitness and preference. MZL subanalyses of larger randomised trials in indolent B-NHL have largely informed clinical practice as there have been few subtype-specific studies in MZL due to the rarity of individual subtypes.

MALT lymphoma

MALT lymphomas can involve any mucosal site and presentation depends on disease location; B symptoms are rare. Gastric MALT is the most common site (33%), followed by intestine (3–9%), salivary glands (16%), orbit (10–12%) and lung (6–10%). MALT lymphomas often remain localised for many years, but disseminated disease at presentation may occur in 25 and 46% of cases of gastric and non-gastric MALTs, respectively, dictating the need for comprehensive staging. Dissemination can be either to other sites of mucosal tissue or to lymph nodes, bone marrow, spleen or liver. Nodal and bone marrow involvement at presentation confers a worse prognosis. MALT lymphomas typically follow an indolent course, but transformation to high-grade disease occurs in about 10% of cases.

Gastric MALT

The pathogenesis of gastric MALT is strongly linked to chronic inflammation secondary to *H. pylori* infection. It typically presents with non-specific upper gastrointestinal tract symptoms (dyspepsia, epigastric pain and bleeding) or anaemia and is diagnosed at endoscopic biopsy. Endoscopic biopsies should be taken from each region of the stomach and duodenum to provide samples for histopathology, cytogenetics to detect the t(11;18) translocation, and examination for *Helicobacter pylori*, which can be confirmed histologically, by urea breath test or by faecal serological testing.

Early-stage gastric MALT can be successfully controlled by *H.pylori* eradication alone, typically consisting of a proton pump inhibitor in combination with amoxicillin and clarithromycin. The approach is most effective for localised disease and when t(11;18) is not detected. Infiltration beyond the mucosa reduces the success of *H. pylori* eradication, irrespective of t(11;18) translocation status, indicating the need for reliable assessment of gastric wall depth in determining the need for additional systemic therapy. Invasive depth is evaluated histologically or by endoscopic ultrasound.

Although more effective for localised tumours with detectable *H.pylori* infection, significant responses have also been reported in *H.pylori* negative and advanced gastric MALT, possibly due to a false negative test, infection by other Helicobacter species, or when disseminated disease is dependent on *H.pylori* for disease progression. For these reasons, current guidelines recommend *H. pylori* triple eradication therapy as an inexpensive initial treatment for all cases of gastric MALT, irrespective of *H. pylori* status, disease stage or t(11;18) status.

Eradication of *H. pylori* induces complete histological remission in approximately 78% of early-stage gastric MALT lymphomas, with 5-year OS exceeding 95%. The median time to histological remission is 6 months, but can take up to 24 months after eradication, advocating the need for endoscopy surveillance every 3–6 months for up to 2 years to confirm eradication of infection and assess for histological regression. Even when histological CR is achieved, a population of monoclonal B-cells remains detectable by PCR in ~50% of patients, but this alone does not necessitate additional treatment. Similarly, no additional therapy is required for patients with histological evidence of residual disease if they are asymptomatic; they too should be managed with active surveillance endoscopies.

Involved site radiotherapy should be considered for localised disease with persistent *H.pylori* infection or symptoms after eradication therapy, which is more likely in tumours genetically driven by the t(11;18) translocation. 30Gy involved field radiotherapy to the stomach is highly effective for patients with stage I/II disease, providing a 5-year disease-free survival of 93%. Relapses are more frequent for tumours located at sites other than the gastric body. The LY03 trial showed no clinical benefit of adding chlorambucil after *H. pylori* eradication.

Rituximab alone or in combination with chemotherapy (discussed below) should be considered for symptomatic patients with localised or advanced-stage disease if eradication therapy has failed and radiotherapy is not an option.

Non-gastric MALT lymphoma

Non-gastric MALT lymphomas can arise at many sites, often in response to chronic infection, inflammation or autoimmune disease. Ocular adnexal MALT lymphoma is linked to infection with *Chlamydia psittaci*, extrandodal MZL of the skin to *Borrelia burgdorferi*, MALT lymphoma of the thyroid to Hashimoto's thyroiditis and salivary gland MALT lymphoma to Sjogren's syndrome. Disseminated disease is more frequently identified in non-gastric than gastric MALT lymphomas.

The management of non-gastric MALT lymphoma depends on site, stage and presence or absence of symptoms. Anti-infective therapy may be effective. An analysis of 7 studies (4 retrospective) comprising 131 patients, mostly with ocular adnexal lymphoma receiving doxycycline for *Chlamydia psittaci*, reported an ORR of 45% at a median follow-up of 25 months. Best responses were achieved in patients with evidence of infection, although interestingly responses were also reported in cases without detectable *Chlamydia psittaci*. Similar responses to antibiotics have not been observed in MALT lymphoma occurring at other sites.

Radiotherapy is effective in early-stage disease, with 25–35 Gy producing local control and long-term freedom from disease in most cases. A recent retrospective study in 68 patients with early-stage non-gastric MALT, predominantly stage I treated with 24–30 Gy involved field or involved site radiotherapy, reported excellent PFS and OS rates of 79 and 95% at a median follow-up of 5 years. Interestingly, in this study auto-antibodies or autoimmune events were detected in 38% of patients, and 24% of patients were *H.pylori* positive.

Patients with advanced-stage symptomatic disease should be considered for systemic therapy (discussed below); radiotherapy retains a role for the management of localised symptoms.

Splenic marginal zone lymphoma

Splenic marginal zone lymphoma (SMZL) is a rare disorder, comprising less than 2% of lymphoid neoplasms. It typically presents with splenomegaly, moderate lymphocytosis and bone marrow involvement with associated cytopenias. B symptoms are present in one-third of patients. Circulating lymphocytes have characteristic cytoplasmic projections, and are termed villous lymphocytes. Splenic hilar lymph nodes may be infiltrated, but peripheral lymph nodes and extranodal sites are usually spared. A paraprotein, usually IgM, is detectable in up to 25% of cases, and autoimmune phenomena occur in 20% of patients, including autoimmune haemolytic anaemia, ITP and cold agglutinin disease. Diagnosis depends on splenic histology or peripheral blood flow cytometry demonstrating typical cell morphology, with an MZL immunophenotype and intrasinusoidal CD20-positive cells in the bone marrow. Features are not unique and diagnosis often requires exclusion of other B-cell NHLs.

There are no pathognomonic chromosomal aberrations associated with SMZL. Pathogenesis is also linked to deregulated genes involved in splenic marginal zone B-cell differentiation (NF-kB signalling genes *NOTCH2* and *KLF2*), and chronic antigenic stimulation evidenced by a highly restricted Ig gene repertoire. Hepatitis C virus (HCV) infection is associated with up to 36% of SMZL cases in areas such as Japan where HCV is endemic.

SMZL generally follows an indolent course, even when the bone marrow is involved, with a median OS of 11.2 years – however ~30% have an inferior outcome and up to 10% transform to high-grade lymphoma.

The HPLL prognostic score was recently proposed and independently validated for lymphoma-specific survival (LSS) in SMZL, incorporating haemoglobin <95 g/L, platelet count $<80 \times 10^9$/L, elevated LDH, and extrahilar lymphadenopathy. Groups A (no risk factors), B (1–2 risk factors) and C (3–4 risk factors) had a 5-year LSS of 95, 87, and 68%, respectively. POD24 was shown to have an independent association with OS in SMZL in a recent retrospective analysis of SMZL with bone marrow involvement.

Therapy is generally reserved for patients with symptomatic or advanced-stage disease, defined by 2008 Splenic Lymphoma Study Group (SLSG) consensus criteria as the presence of one or more of the following: symptomatic cytopenias, progressive or bulky splenomegaly, rapidly rising lymphocyte count,

B symptoms and/or involvement of nodal or extranodal sites >2 cm. Patients with early/asymptomatic SMZL, characterised by the absence of these features, may be managed with active surveillance, with up to 50% of asymptomatic patients never requiring treatment.

For decades, splenectomy has been the principal treatment for symptomatic patients or those with cytopenias, providing patients are fit for surgery and there is no nodal dissemination beyond the splenic hilum. ORR to splenectomy ranges from 60 to 100%, with a median duration of response (DOR) of 4 years, 5-year PFS 35–61% and median TTNT of 8 years. Splenectomy resolves cytopenias and improves quality of life ~90% of patients but does not impact bone marrow infiltration or lymphocytosis. Splenic irradiation may be an option for patients with poor performance status or as a palliative measure. There may also be a role for interferon-α (IFN), with or without ribavirin, in patients with SMZL and concurrent HCV infection. Such patients may achieve durable partial and occasional complete remissions, and new IFN-free regimens with direct-acting antiviral agents are in development.

Rituximab monotherapy produces similar outcomes to splenectomy and is increasingly used instead of splenectomy and in those unsuitable for this approach due to significant cytopenias. A retrospective evaluation of rituximab induction in 108 patients meeting SLSG criteria produced very high responses (ORR 92%), mostly CR/CRu (CR unconfirmed) (65%), with 10 year FFP and OS rates of 64 and 85%, respectively. A maintenance phase of rituximab for 1–2 years improved 9-year FFP from 42 to 76%, with no impact on survival or PFS advantage for 2 versus 1 year of maintenance.

Rituximab in combination with chemotherapy should be considered for patients with symptomatic progressive disease after splenectomy, rituximab monotherapy or anti-viral therapy (discussed later).

Nodal marginal zone lymphoma

There are no specific guidelines for managing nodal marginal zone lymphoma (NMZL) and recommendations tend to follow those for FL, i.e. in localised disease, radiotherapy is effective and may be curative, in advanced-stage asymptomatic disease, a watch-and-wait approach is acceptable, whilst in symptomatic advanced-stage disease, chemotherapy in combination with rituximab is frequently used.

First-line systemic therapy for symptomatic advanced-stage MZL

Systemic therapy should be considered for symptomatic advanced-stage disease or localised disease that has failed appropriate local therapy or antibiotics. Several trials have demonstrated the value of 'light touch' rituximab or chlorambucil monotherapy and low-intensity immunochemotherapy (R-chlorambucil and RCVP), especially for MALT and SMZL histologies. Table 26.4 summarises the key first-line trials of immunotherapy and immunochemotherapy for advanced stage MZL.

IELSG-19, a randomised trial in gastric and non-gastric MALT lymphomas (including 40% stage l patients who had failed prior local therapy), demonstrated excellent outcomes for monotherapy using rituximab or chlorambucil; ORR 78–85% and median EFS >5 years. Combining these agents improved ORR (to 95%) and EFS (not reached), but 5-year survival was universally excellent (89–92%) with no difference between treatments. In a phase 2 study in advanced MZL, first-line RCVP also produced high ORR and 3-year PFS of 88 and 59% in MALT and NMZL lymphomas.

The benefits of bendamustine over other chemotherapies are less clear in MZL as it is for other low-grade B-NHLs. Two small single-arm phase 2 trials identified promising activity for BR in MALT lymphomas (MALT2008-01, $n = 60$), including patients with localised antibiotic-resistant gastric MALT, and SMZL (IELSG36 BRISMA, $n = 56$). These trials reported very high response rates (90–100%), EFS was 88% at 4 years for MALT lymphomas and PFS was 90% at 3 years for SMZL. It should be noted that over half of patients in the BRISMA trial had low-risk (HPLL Group A) disease, and neither studies were randomised, making it difficult to ascertain if BR is superior to rituximab monotherapy or less intensive immunochemotherapy. Subset analyses of MZL enrolled on the randomised BRIGHT ($n = 46$) and StiL 2003 ($n = 67$) trials in indolent B-NHL reported a trend towards higher response rates for BR compared to RCHOP/RCVP, but there was no difference in PFS for BR versus RCHOP. These results should be interpreted with caution in these small, inadequately powered analyses.

The GALLIUM randomised trial of obinutuzumab versus rituximab in combination with CVP, CHOP or bendamustine (B) demonstrated no advantage for obinutuzumab in a subset analysis of 195 enrolled MZL patients (nodal, 66; extranodal, 61; splenic, 68), most of which (83%) had advanced stage disease. Both antibodies produced high 3-year PFS rates of 75–78%, but obinutuzumab was associated with a higher frequency of high-grade toxicity (82 versus 77%) and fatal adverse events (11.9 versus 6.5%). Obinutuzumab should not be combined with chemotherapy for treatment of MZL.

Fifty-eight percent of MZL patients in the afore-mentioned GALLIUM trial received maintenance therapy; however, the role of maintenance therapy in MZL is an evolving question. A non-randomised phase 2 trial from the CISL group demonstrated a 20% improvement compared to historic controls for 2 years of maintenance after RCVP (3y PFS 81 versus 59%). The randomised MAINTAIN trial demonstrated a superior 2-year PFS for maintenance versus no-maintenance after BR (not reached versus 92 months, $p = 0.008$) at a median follow-up of

Table 26.4 Summary of results of key trials of immunotherapy and immunochemotherapy in the first-line treatment of advanced-stage marginal zone lymphoma.

First author, year of publication (trial)	Treatment, *n*	Subtype, *n*	Median follow-up (months)	ORR, CR (%)	PFS/EFS (%)	OS (%)
Herold, 2017 (GALLIUM)	R-chemo, 96 O-chemo, 99 (both arms with maintenance)	NMZL, 66 MALT, 61 SMZL, 68	38	81, 19 83, 16	PFS 75 at 3 yrs PFS 78 at 3 yrs (ns)	Not reported
Flinn, 2014 (BRIGHT)	BR, 28 RCHOP/RCVP, 18	All subtypes, not specified	Not reported	92, 20 71, 24 (ns)	Not reported	Not reported
Rummel, 2013 (STiL NHL1)	BR, 37 RCHOP, 30	All subtypes, not specified	45	93, 40 91, 30 (ns)	Median 57 month median 47 month (ns)	Not reported
Rummel, 2018 (MAINTAIN)	BR, 61 BR + maintenance, 59	NMZL + SMZL, not specified	76	91, 19	Median 92 month Not reached ($P = 0.008$)	86 at 6 yrs 92 at 6 yrs (ns)
Ianitto, 2018 (BRISMA)	BR, 56	SMZL	32	91, 73	PFS 90 at 3 yrs	96at 3 yrs
Salar, 2017 (MALT 2008-01)	BR, 57	MALT	82	100, 75	EFS 87 at 7 yrs	96 at 6 yrs
Kang, 2012	RCVP, 40	NMZL, 12 MALT, 28	53	88, 60	PFS 59 at 3 yrs	83 at 4 yrs
Oh, 2019	RCVP + maintenance, 45	NMZL, 15 MALT, 30	38	93, 44	PFS 81 at 3 yrs	90 at 3 yrs
Zucca, 2017 (IELSG-19)	R, 138 Chlorambucil, 131 R-Chlorambucil, 132	MALT	89	78, 56 86, 63 95, 79	EFS 50 at 5 yrs EFS 51 at 5 yrs EFS 68 at 5 yrs	90 at 5 yrs

BR, bendamustine, rituximab; CR, complete response; EFS, event-free survival; MALT, mucosa-associated extranodal MZL; mo, months; NMZL, nodal MZL; ns, not statistically significant; O, obinutuzumab; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; R, rituximab; R-chemo, RCVP, RCHOP, BR; O-chemo, O-CVP, OCHOP, OB; RCHOP, rituximab, doxorubicin, cyclophosphamide, vincristine, prednisolone; RCVP, rituximab, cyclophosphamide, vincristine, prednisolone; SMZL, splenic MZL; yrs, years.

76 months. No difference in OS was observed between the groups. Final results of this trial are awaited; however, these early data suggest that maintenance therapy may confer a PFS (but not OS) advantage in MZL patients and could be considered for selected patients.

Management of relapsed MZL

Treatment pathways for relapsed MZL are similar to relapsed FL pathways. Immunochemotherapy has historically been the therapy of choice, resulting in ORR rates of 85–93% and CR rates of 54–78%. Many patients with MZL are elderly or experience excess toxicities with standard approaches, leading to considerable interest in novel approaches. Single-agent rituximab, chlorambucil or R-chlorambucil are options for relapsed MALT lymphomas based on the IELSG-19 trial, which enrolled a minority (8%) of patients with relapsed/refractory MZL; interestingly, their OS was similar to first-line patients. BR is very active in relapsed MALT, achieving ORR rates of 93%, but at a cost of almost 30% grade 3–4 haematological toxicity. Bortezomib is also active, achieving ORR 48%, CR 31%. Patients with relapsed/refractory SMZL (including those who progress after splenectomy or rituximab monotherapy) may have rituximab re-treatment or immunochemotherapy. Patients with relapsed/refractory NMZL follow similar pathways to relapsed/refractory FL.

Bruton's tyrosine kinase (BTK) plays a critical role in the pathogenesis of MZL, and several BTK inhibitors have been developed in relapsed/refractory MZL. A pivotal phase 2 trial of ibrutinib monotherapy led to FDA approval based on an ORR of 48% (although CRs were rare at 2%) and median PFS of 14.2 months, with 38% of patients still on treatment at 19 months. Outcomes were best for SMZL and MALT. In the recently completed MAGNOLIA trial, zanubrutinib (a second-generation BTK inhibitor) demonstrated very promising activity and was granted FDA priority review based on an ORR of 74%, CR 24% and 9 month PFS of 67%. Results were even better in high-risk subsets, including patients aged over 75 years (ORR 89%), refractory to last therapy (ORR 71%) or those with NMZL (ORR 84%). Zanubrutinib also had a favourable safety profile compared to other novel agents

in development and has now received a European and UK license. It is likely to have a key future role in the management of relapsed/refractory MZL.

Lenalidomide in combination with rituximab (R2) is an FDA-approved option for relapsed/refractory MZL based on data from the AUGMENT and MAGNIFY trials. Interestingly, adding lenalidomide to rituximab improved ORR (65 versus 44%) but not PFS compared to rituximab alone in the MZL subset analysis of the AUGMENT trial, attributed to the small size and imbalance in baseline characteristics. R2 does not have approval for relapsed/refractory MZL in the UK and EU.

Other novel agents with emerging activity in MZL include the PI3k inhibitors (although most have now been withdrawn from the market) and axicabtagene ciloleucel; but whilst the latter has demonstrated promising utility, a higher rate of CRS and ICANS was reported for MZL compared to FL suggesting the need for and judicious patient selection when considering these experimental therapies. Similarly, CD3–CD20 bispecific antibodies may be expected to be active in MZL but higher rates of CRS may be anticipated especially in SMZL, based on emerging safety data.

Waldenström macroglobulinaemia

Waldenström macroglobulinaemia (WM) is a clinical syndrome that occurs in patients with clonal proliferation of post-germinal-centre IgM-secreting lymphoplasmacytic cells in the bone marrow or other organs and a detectable IgM paraprotein. Lymphoplasmacytic cells express IgM, CD19 and CD20 and a mutation in the *MYD88* gene – *L265P* – leading to downstream activation of the BTK pathway and activation of NFκB and other pathways in >90% of cases. Mutations in *MYD88* also occur in a small proportion of MZL cases. *CXCR4* is mutated in ~30% of cases. The presence or absence of *MYD88* and *CXCR4* mutations are increasingly used to predict response and choice of therapy.

WM is mainly a disease of the elderly, with a median age at presentation exceeding 70 years, more common in males, and an incidence in the UK of 0.55/100,000 per year. With modern therapeutic approaches, WM has a median survival from diagnosis of over 8 years.

Like other low-grade lymphomas, WM may be asymptomatic at diagnosis but wide-ranging symptoms and clinical manifestations can occur due to bone marrow infiltration, lymphadenopathy and IgM paraproteinaemia. High IgM levels >50 g/L may cause hyperviscosity syndrome, with symptoms including fatigue, headache, blurred vision, mucosal bleeding, impaired cognition or reduced level of consciousness requiring urgent management. Other clinical manifestations include haemostatic abnormalities, polyneuropathies (which may be related to anti-myelin-associated glycoprotein (MAG) antibodies), cryoglobulinaemia, cold agglutinin haemolytic anaemia (CHAD), acquired von Willebrand's disease and, rarely, consequences of light-chain tissue deposition such as amyloidosis. Approximately one-third of patients present with lymphadenopathy, splenomegaly or hepatomegaly. Rarely, there is direct central nervous system (CNS) involvement, termed Bing Neel syndrome (BNS), occurring in ~1% of patients. The clinical manifestations of BNS are varied and can occur at any point in the disease course. It is important to distinguish BNS from the more common peripheral neuropathy seen in patients with WM. There are no established diagnostic criteria for BNS; leptomeningeal enhancement on magnetic resonance imaging (MRI) is common but not essential, and cerebrospinal fluid (CSF) examination typically reveals WM cells with the *MYD88* mutation.

Table 26.5 Risk stratification in Waldenström macroglobulinaemia according to the International Prognostic Scoring system for WM.

Risk group	Number of risk factors	Median survival (months)
Low	0–1 (except age)	143
Intermediate	Age (alone) or any two risk factors	99
High	3–5	44

Risk factors: Age >65 years, haemoglobin ≤115 g/L, platelet count ≤100 × 10^9/L, β2M >3 mg/L, monoclonal IgM >70 g/L.

WM is risk stratified according to the International Prognostic Staging System for WM (IPSSWM), which defines three risk groups based on age, haemoglobin, thrombocytopenia, paraprotein level and β2M (see Table 26.5). Of note, data used to generate this risk score included historically treated patients and median survival may be longer for patients diagnosed today.

Response in WM is based on the International Workshop on WM (IWWM) guidelines and includes assessment of serum paraprotein, bone marrow infiltration, lymphadenopathy/organomegaly (if present at baseline), as well as assessment of lymphoma signs and symptoms. Assessment of bone marrow is particularly important and serum IgM alone should not be used to assess response. Responses can be delayed in WM and the timing of response assessment needs to be carefully considered.

Management of Waldenström macroglobulinaemia

Active surveillance is appropriate for asymptomatic patients, but most patients eventually require treatment. In one study, 59% of patients progressed within 5 years, and 75% eventually required treatment. Indications for treatment defined by the IWWM include constitutional symptoms, symptomatic

lymphadenopathy or splenomegaly, hyperviscosity syndrome, cytopenias due to marrow infiltration and IgM-related syndromes (including CHAD and neuropathy). Anaemia is the most common indication for treatment. Patients presenting with hyperviscosity should be considered for urgent plasmapheresis.

There is a range of treatment options for patients with symptomatic WM, albeit with relatively few randomised controlled trials to guide choice and sequencing. With many new therapies emerging, individual therapy choices depend on patient factors and comorbidities, disease factors (such as symptoms, risk factors and mutational status of *MYD88* and *CXCR4)* and availability of treatments.

Previously, alkylating agents such as chlorambucil were the mainstay of treatment for symptomatic WM, producing remissions in 50–75% of patients. Subsequent studies demonstrated that rituximab monotherapy was effective, achieving responses in up to 60% of previously untreated patients with a median PFS of 14–24 months and a favourable side effect profile compared to alkylator therapy. Rituximab can cause a sudden rise in serum IgM and viscosity in some patients, so-called 'IgM flare', therefore close monitoring for hyperviscosity is recommended and rituximab should be avoided when IgM exceeds 40 g/L.

Later studies demonstrated that combining rituximab with chemotherapy deepens remissions compared to either chemotherapy or rituximab alone. R-fludarabine, RCHOP, R-cyclophosphamide-dexamethasone (DRC) and BR all demonstrated response rates >90% with major response rates >75% and durable remissions. BR achieves a median PFS of 65 months and is often favoured due to its efficacy and favourable toxicity profile. In patients with a high paraprotein concentration, rituximab is deferred until the second or third cycle to mitigate IgM flare; alternatively, pre-emptive plasmapheresis may be used alongside systemic therapy. Response to immunochemotherapy does not seem to be related to *MYD88* or *CXCR4* mutational status.

Evidence from previous retrospective series suggested that maintenance rituximab after immunochemotherapy improves outcomes but a randomised trial of maintenance or observation in patients achieving at least PR to BR found no significant difference in PFS or OS. Accordingly, maintenance rituximab should no longer be considered for these patients.

Proteasome inhibitors such as bortezomib are effective in combination with rituximab, with or without dexamethasone. Treatment-emergent peripheral neuropathy is partially mitigated by administering bortezomib subcutaneously but care should be taken in patients with pre-existing neurological manifestations of WM. Newer proteasome inhibitors, carfilzomib and the orally available ixazomib, have been assessed in combination with rituximab and dexamethasone, demonstrating high activity with different safety profiles.

Several recent non-randomised trials showed that the oral BTK inhibitor, ibrutinib, is highly active in WM leading to EU and FDA licensing for patients with symptomatic disease. In these trials, ibrutinib monotherapy achieved responses in >90% of patients whether used upfront or at relapse, with a major response in >70% of patients, often durable, and 5-year PFS >50% in one trial. Ibrutinib monotherapy is less effective in patients with wild-type *MYD88* and mutated *CXCR4*; immunochemotherapy may be preferred in these patients.

Rituximab plus ibrutinib has also been assessed but not yet directly compared with ibrutinib monotherapy. This combination produces rapid and deep remissions and may also be effective in patients without *MYD88* mutation. Ibrutinib crosses the blood–brain barrier with case reports of efficacy in BNS. Important toxicities of ibrutinib include bleeding, atrial fibrillation, hypertension and respiratory tract infections. Several other BTK inhibitors are in development; these have different side effect profiles and may be more effective, but carefully conducted trials are needed to understand their role in the treatment pathway.

Management of relapsed WM depends on prior therapy, duration of remission and patient fitness. It is reasonable to retreat with the same regimen provided there was a good response previously and if retreatment is funded. An alternative regimen should be considered for patients with short previous remissions (<1 year).

Stem cell transplantation for Waldenström macroglobulinaemia

The role of stem cell transplantation in WM remains unclear with a lack of high-quality evidence to guide its use. Autologous SCT may be considered in second or subsequent relapses in high-risk patients, although it is now generally considered that SCT should not be offered to BTKi-naïve patients. There is evidence to suggest a graft-versus-disease effect in WM and, therefore, allogeneic HSCT may be appropriate in selected younger patients, with a 5-year PFS of 49–56% and OS of 62–64% reported. The 3-year non-relapse mortality was 33% after myeloablative conditioning and 23% after reduced-intensity conditioning in the largest registry series reported to date. The emergence of many new treatment options in WM makes it difficult to define the place of SCT in the contemporary management of WM. IWWM guidelines for managing WM are summarised in Table 26.6.

New approaches in WM

As with other low-grade B-cell NHLs, many new treatment approaches are in development and the treatment landscape is likely to change in coming years. Agents under investigation in WM include the BCL2 inhibitor venetoclax, second and later-generation BTK inhibitors, CXCR4 antagonists, CD3-CD20 bispecific antibodies and CAR-T cell therapy. Carefully conducted, academic clinical trials with international

Table 26.6 Guidelines for management of Waldenström macroglobulinaemia.

The IWWM published updated recommendations for treatment WM in 2020 to incorporate ibrutinib in first-line treatment options. Suggestions for the management of WM, taking into account the 2020 iteration of the IWWM recommendations, are presented below whilst acknowledging that choice of therapy will depend on local availability:

- First-line treatment for symptomatic patients should be BR, bortezomib-rituximab-dexamethasone, cyclophosphamide-dexamethasone-rituximab, or ibrutinib (+/− rituximab). For patients who are $MYD88^{WT}$ or $CXCR4^{mut}$ ibrutinib alone is unlikely to be effective – inrutinib with rituximab or an immunochemotherapy regimen should be considered instead.
- CHOP and purine analogue based treatments are not recommended in most cases.
- Avoid bortezomib in patients with neuropathy
- Avoid carfilzomib in patients with cardiac disease or age >65 years
- Avoid purine analogues in patients who may be stem cell transplant candidates
- Patients with relapsed disease can be treated with an alternative first-line regimen or with the same regimen if previous duration of response was >1 year.
- Autologous SCT may be considered for patients with high risk, refractory or relapsed disease if they have previously had BTKi.
- Allogeneic transplantation should usually only be undertaken in the context of a clinical trial.
- Plasmapheresis should be considered as interim therapy until definitive therapy can be initiated.
- Patients with WM should be enrolled into clinical trials whenever possible.
- Patients should be stratified according to the IPSSWM.
- Response assessment should be according to the IWWM criteria.

collaboration are necessary to understand which of these therapies are most beneficial, how best to sequence them, and to determine the optional duration of treatment.

Mantle cell lymphoma

Mantle cell lymphoma (MCL) comprises approximately 5% of all non-Hodgkin lymphomas (NHLs). It typically occurs in older patients with a median age at diagnosis of 65 years and is more common in males. The natural history and prognosis of MCL are highly variable ranging from truly indolent disease with a long overall survival and no need for urgent therapy to a rapidly progressive illness requiring prompt intervention. Relapses are inevitable with contemporary strategies but, as with the other B-NHLs, the myriad of emerging therapeutic approaches stands to change the treatment paradigm. The median overall survival was historically only 3–5 years (substantially shorter than other low-grade B-NHLs) but with modern approaches median OS exceeding 8 years has been reported for intensively treated patients. Further improvements are anticipated with the advent of targeted treatments.

Most cases of MCL are characterised by the t(11;14)(q13;q32) translocation leading to over-expression of cyclin D1 and cell-cycle dysregulation. In rare cases, cyclin D1 is not expressed. Upregulation of the neural transcription factor SOX11 has been identified in 90% and may be helpful in diagnosing cyclin D1 negative cases.

Presentation with advanced-stage disease including extranodal involvement occurs in >90% of cases, commonly involving the bone marrow and gastrointestinal tract. CNS involvement is rare at presentation but may occur at relapse. MCL is typically FDG-avid and therefore FDG-PET scanning is frequently used for staging and response assessment.

Two distinct subtypes of MCL were defined in the 2016 revised WHO classification of lymphoid malignancies: nodal MCL (80–90%) and non-nodal MCL (10–20%). In nodal MCL, IGH is unmutated, SOX11 is over-expressed and the disease typically behaves aggressively. Blastoid MCL is a variant of nodal MCL with a high proliferation rate and aggressive clinical course. Non-nodal MCL is characterised by a leukaemic presentation with splenomegaly, IGH is mutated and SOX11 absent. Non-nodal MCL typically follows an indolent trajectory.

Prognosis of MCL

Patients with MCL can be stratified into three main prognostic groups according to the MCL International Prognostic Index (MIPI), which incorporates age, performance status, LDH and leucocytosis. Low-risk MIPI patients have a median OS >5 years, whilst OS in intermediate- and high-risk MIPI groups is only 51 and 29 months, respectively. Additional factors affecting prognosis include Ki67 score, β2M and CNS involvement. Ki67 was incorporated into the MIPI score to give a 'biological MIPI' (MIPIb). Despite significant inter-observer variability in Ki67 quantification, a high proliferation index is widely accepted as a poor prognostic factor. *TP53* mutations occur in ~10% of cases at diagnosis and have also recently been identified as a strong predictor of poor outcome in MCL that may be used to direct treatment strategies. Patients with *TP53* mutations often have other high-risk features (high Ki67 and high MIPI). Minimal residual disease (MRD) assessment was also found to be highly predictive of response in several trials and may in

future be used to tailor or adapt therapy, but has not yet been widely adopted in routine clinical practice.

First-line management of MCL

The management of MCL is undergoing rapid change, as increased understanding of disease pathogenesis is leading to molecularly targeted therapies.

Whilst most patients require treatment at presentation, ~25% of patients may initially be observed, with retrospective data showing that this delays initiation of therapy for a median of 12 months. This approach is most often applied for non-nodal MCL (see above), but patients with asymptomatic nodal presentations may also be observed initially.

The choice of first-line treatment depends on patient factors (age, fitness and comorbidities), and assessment of disease features (symptoms, MIPI and *TP53* status). The most important assessment is whether or not the patient is fit for autologous stem cell transplantation (ASCT).

If fit for ASCT, treatment with rituximab in combination with chemotherapy followed by consolidation ASCT is appropriate. For this fit population, there is no agreed international standard of care but incorporation of cytarabine, an anthracycline and rituximab are considered essential.

In the second Nordic Mantle Cell Lymphoma trial (MCL2), 160 patients were treated with dose-intense R-CHOP (R-maxi-CHOP), alternating with cycles of rituximab plus high-dose cytarabine, followed by consolidation ASCT. The CR rate after ASCT was 90% and with a median follow-up of 11 years, median OS and PFS were 12.7 and 8.5 years, respectively. The success of this regimen may be due to the intensive use of cytarabine and *in vivo* purging with rituximab prior to stem cell harvest. Despite never having been assessed in a prospective randomised trial, this approach is still widely followed for patients considered fit for intensive treatment.

A similar approach is six cycles of alternating R-CHOP/DHAP (rituximab plus dexamethasone, high-dose cytarabine and cisplatin) and consolidation ASCT, shown in the randomised 'MCL Younger trial' to be superior to R-CHOP followed by ASCT. The R-CHOP/DHAP and MCL2 (Nordic) approaches have not been compared in a randomised trial and the choice between these two similar regimens is largely determined by centre preference.

Another approach for fit patients is rituximab, hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone, alternating with high-dose methotrexate and cytarabine (R-Hyper-CVAD). This regimen is used without consolidation ASCT and achieves very high response and survival rates in phase 2 studies, but it is associated with significant toxicities limiting its widespread use.

Although many studies have shown that patients with *TP53* mutations have significantly worse outcomes with intensive upfront treatment approaches there is as yet no internationally agreed approach for treating such patients. Wherever possible such patients should be entered into trials of novel approaches.

Rituximab maintenance every 2 months for 3 years after consolidation ASCT significantly prolongs PFS (4-year PFS 83 versus 64%) and OS (4-year OS 89 versus 80%, HR 0.5) and should be offered as standard of care unless contraindicated.

There are a number of first-line treatment options for patients who are unfit for ASCT, with no established gold standard. The European MCL network trial demonstrated that R-CHOP was superior to R-FC (rituximab, fludarabine and cyclophosphamide), partly due to lower toxicity. The StiL trial demonstrated a PFS advantage for BR over R-CHOP in 94 patients with MCL, with a median PFS of 35 versus 22 months; therefore, BR is often used in this cohort. Adding cytarabine to bendamustine and rituximab (R-BAC) is very effective but relatively intensive in this older population and requires judicious patient selection. If the proteasome inhibitor bortezomib is available, it can be combined with rituximab, cyclophosphamide, doxorubicin and prednisolone in the VR-CAP regimen, which has demonstrated improved OS over R-CHOP albeit maintenance rituximab was not given after induction. Single-agent rituximab has low efficacy in MCL and should only be considered if chemotherapy is contraindicated.

The European MCL network trial for elderly patients included a second randomisation to rituximab or interferon maintenance and reported both a PFS and OS benefit for rituximab maintenance. Rituximab maintenance should therefore be considered in patients who have responded to induction treatment but are unfit for more intensive therapies. Although maintenance has only been assessed after R-CHOP, it is widely applied after varied induction regimens.

Many new therapies for MCL are being assessed and it is likely that firstline treatment options will expand in coming years. For example, ibrutinib in combination with rituximab is being assessed in first-line treatment of MCL in older pts in a UK-NCRI randomised trial against R-bendamustine or R-CHOP.

Relapsed disease and novel treatments

There are no standard treatments for relapsed MCL. Treatment decisions are based on duration of response to prior treatments, disease characteristics e.g. *TP53* status, performance status, drug availability and patient preference. Therapeutic options in relapsed disease are changing, with the introduction of numerous targeted therapies; some of which are now also being investigated in the first-line setting. Wherever possible, patients with relapsed disease should be enrolled in clinical trials.

The BTK inhibitor ibrutinib, now licensed in relapsed or refractory MCL, delivers an ORR of ~65% and median PFS of 12–13 months when used as monotherapy. Median OS is however short at only 25 months and very short for those who progress on treatment, indicating the challenges of relapsed disease. Ibrutinib is more effective when used early in the relapsed pathway with superior PFS and OS in second line compared to later

lines in a large retrospective analysis. Accordingly, for many patients, ibrutinib should be considered at second line. Ibrutinib may be combined with rituximab to deliver potentially superior disease control. Whether used as monotherapy or in combination with rituximab, outcomes are inferior for patients with *TP53* mutation and high Ki67.

Second-generation BTK inhibitors acalabrutinib and zanubrutinib have received FDA approval for relapsed MCL and have a more favourable side effect profile than ibrutinib. Head-to-head comparisons have not been performed to compare efficacy. Other BTK inhibitors in development include pirtobrutinib, a highly selective non-covalent BTK inhibitor with very promising results in early-phase trials. Understanding how to combine these drugs with other active agents in MCL in both relapsed and frontline treatment is a major research area.

If a BTK inhibitor is not available or a patient has progressed after BTKi there are a number of other options to consider. Bortezomib is active as monotherapy or in combination with chemotherapy such as CHOP or R-CHOP in the relapsed setting. Lenalidomide is also effective either as monotherapy or in combination with rituximab delivering response rates of 35–50% and some durable remissions. Lenalidomide in combination with BTK inhibitors and other agents is under investigation. Venetoclax has very high single-agent activity achieving response rates of 75%, including patients who have failed BTK inhibitors.

CAR-T cell therapy with brexucabtagene autoleucel (formerly KTE-X19; Tecartus) was approved in 2021 for relapsed MCL based on results of the ZUMA-2 trial. In this trial of 71 patients very high response rate of 85% (CR 59%) were achieved, with 12-month PFS and OS rates of 61 and 83%, respectively. These impressive results led to approval of Tecartus. Mature follow-up will determine if initial response rates translate into long-term disease control. Other CAR-T cell products are in development and it is hoped that toxicities will be more manageable to allow this treatment to be delivered to older, frailer patients with comorbidities. Efforts to incorporate CAR-T therapies into the MCL treatment pathway should focus on those who have progressed after BTK inhibitors, especially those with adverse risk factors such as *TP53* mutation.

Reduced-intensity allogeneic stem cell transplantation can be considered for young, fit patients with relapsed disease and high-risk features. A retrospective analysis of reduced-intensity allogeneic HSCT for 70 MCL patients reported to BSBMT showed a 5-year relapse risk of 67%, PFS of 14% and OS of 37% with a TRM of over 20% at 1 year.

Given the numerous new treatment options in MCL, it is clear that wherever possible, patients should be enrolled into clinical trials to understand how best to combine and sequence these exciting new treatments to improve outcomes for patients with MCL. Focusing efforts on risk stratification and improving outcomes for those with high-risk disease is a priority.

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CHAPTER 27

Aggressive B-cell non-Hodgkin lymphoma

27

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Introduction

The term aggressive (often termed high-grade) non-Hodgkin lymphoma (NHL) originally referred to one of three categories defined by the International Working Formulation (IWF) classification system introduced in 1981 and included just three histological subtypes: large cell immunoblastic lymphoma, lymphoblastic lymphoma and Burkitt lymphoma. Today a number of clonal heterogeneous pathological entities fall under this broad umbrella term describing lymphomas that are highly proliferative. The term 'aggressive lymphoma' is now preferred to 'high-grade lymphoma' as since 2016, high-grade B-cell lymphoma is a specific entity, distinct from other aggressive lymphomas. Aggressive lymphomas exhibit distinct epidemiology, aetiology, pathogenesis, morphologic, immunophenotypic and clinical features. Paradoxically, despite the aggressive nature of the disease, a significant number of patients can be cured with intensive combination immunochemotherapy regimens. Recently, significant strides have been made in our understanding of the molecular pathogenesis of lymphomas and how this can be potentially translated into novel therapeutic strategies.

Aggressive NHL can also be broadly subcategorised into B-cell and T-cell disorders, depending on the immunophenotype of the malignant clone. This chapter will focus on the pathogenesis, clinical diagnoses and management of aggressive B-cell NHL. Common T-cell lymphomas are covered in Chapter 24.

Epidemiology

Aggressive B-cell NHL represents approximately 45% of all NHL diagnoses with diffuse large B-cell lymphoma (DLBCL) being the most common. The annual incidence rate varies markedly with lymphoma subtype and age, some being more prevalent in children and younger adults whilst others are more common in elderly individuals. The overall incidence of NHL has increased in the Western World over recent decades, rising by 39% in the UK between 1993–1995 and 2015–2017. This rise is in part explained by improved diagnostic techniques but is predominantly driven by increased life expectancy and a significant rise in incidence in older adults. There remains a slight male predominance.

Classification of aggressive B-cell lymphomas

The classification of aggressive B-cell lymphoma is dependent on morphological, immunophenotypic, genetic and molecular features, which form the basis of the World Health Organisation (WHO) classification system (Table 27.1). The most recent version of this classification in 2022 clarified a number of new entities as well as revising existing lymphoma subtypes to reflect increased understanding of pathogenesis and advancement of molecular diagnostics. Precise subtype classification is discussed

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 27.1 Classification of aggressive, mature B-cell lymphomas in accordance with WHO 2022 classification.

Diffuse large B-cell lymphoma (DLBCL), NOS
Germinal-centre B-cell type
Activated B-cell type
Large B-cell lymphoma with IRF4 rearrangement
T-cell/histiocyte-rich large B-cell lymphoma
Primary cutaneous DLBCL, leg type
EBV+ diffuse large B-cell lymphoma
DLBCL associated with chronic inflammation
Fibrin-associated large B-cell lymphoma
Lymphomatoid granulomatosis
Fluid overload-associated large B-cell lymphoma
Primary mediastinal large B-cell lymphoma
Primary large B-cell lymphoma of immune-privileged sites:
Primary large-B cell lymphoma of the central nervous system (CNS)
Primary large B-cell lymphoma of the vitreo/retina
Primary large B-cell lymphoma of the testis
Intravascular large B-cell lymphoma
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV8+ diffuse large B-cell lymphoma
Burkitt lymphoma
High-grade B-cell lymphoma lymphoma with 11q aberrations
Diffuse large B-cell lymphoma/high-grade B-cell lymphoma, with *MYC* and *BCL2* rearrangements
High-grade B-cell lymphoma, NOS
Mediastinal grey zone lymphoma
Transformations of indolent B-cell lymphomas

in detail in Chapter 20. Although most aggressive B-cell lymphomas fall into well-defined categories, there remain subgroups of patients with intermediate or overlap features that present a diagnostic and therapeutic challenge. Ongoing developments in the molecular basis of these disorders will likely refine the current classification system in the future.

Many of the subtypes share the same principles with regard to diagnosis and management. The bulk of this chapter will focus on the most common subtype, diffuse large B-cell lymphoma not otherwise specified (DLBCL, NOS). There will also be short sections describing other subtypes.

Aetiology

With the exception of a handful of aggressive NHL subtypes, the causative factors that underpin lymphomagenesis are unknown. Certain infectious agents are known to play a role in the pathogenesis of specific high-grade lymphomas such as Epstein–Barr virus (EBV) in Burkitt lymphoma, post-transplant lymphoproliferative disorders and plasmablastic lymphoma or human herpesvirus-8 (HHV8) in primary effusion lymphoma and multicentric Castleman's disease. Immunosuppression as a result of human immunodeficiency virus (HIV) or after allogeneic stem cell or orthotopic transplantation is a well-recognised risk factor. Familial aggregation of high-grade lymphomas has rarely been reported suggesting there is no clear genetic susceptibility. Scant epidemiological studies have implicated occupational exposure to insecticides and pesticides as a risk factor in the development of specific lymphomas. However, the exact impact of these factors and their putative role in contributing to lymphomagenesis has not been clarified.

Diagnosis

The diagnosis of aggressive B-cell NHL is made based on a combination of clinical features, radiological assessment, tissue histology and, more recently, molecular confirmation.

Clinical features

Aggressive B-cell NHLs present in a variety of ways. Localised or widespread painless lymphadenopathy is often evident and may be accompanied by B symptoms (unexplained fever of 38 °C or higher, drenching night sweats and loss of more than 10% of body weight in 6 months). Given the rate of tumour proliferation, patients can present with compression symptoms and/or organ dysfunction due to rapidly enlarging lymphadenopathy or extra-nodal masses. Superior vena cava syndrome caused by a bulky mediastinal mass is also more frequent in certain subtypes of NHL such as primary mediastinal B-cell lymphoma. Neurological presentation due to CNS infiltration is typically seen with Primary CNS lymphoma (PCNSL) but rare in systemic lymphomas, although not an uncommon feature of Burkitt lymphoma. Unlike indolent NHL, bone marrow involvement is less common, occurring in less than 20% of cases and has been associated with poorer outcomes. Patients can present with symptoms secondary to deranged haematological or biochemical parameters such as anaemia and hypercalcaemia.

Laboratory investigations

Histological diagnosis from either excision or core biopsy of a lymph node, bone marrow or extranodal mass is essential. Core biopsies are increasingly used in routine clinical practice due to improved radiologically guided sampling techniques and low procedural complications, but excision is sometimes required to obtain larger tissue sections in complex cases. Fine-needle aspiration (FNA) is rarely performed now and can lead to a delay in

diagnosis due to the inadequate tissue obtained, except in suspected cases of Burkitt lymphoma, when a diagnosis can be made on the morphology and immunophenotype, and therapy can be urgent. Immunohistochemistry is routinely performed on tissue sections of lymph nodes and, increasingly, cytogenetic immunoglobulin rearrangement and gene mutation testing.

Anaemia at diagnosis is usually indicative of widespread disease and may reflect a non-specific manifestation of malignancy. However, it can also be due to hypersplenism or bone marrow infiltration. The white count is variable. Overspill of lymphoma cells into the blood can occur in aggressive lymphoma, representing the so-called 'leukaemic phase' but this is infrequent and usually seen in later stages of disease. Hypoalbuminaemia is another feature associated with a systemic disturbance and is indicative of a poor prognosis. A raised level of lactate dehydrogenase (LDH) is usually associated with advanced disease and was identified as an independent prognostic factor in 1993 (within the International Prognostic Index) and remains so today (Table 27.2).

Table 27.2 Clinical prognostic scoring systems in patients with DLBCL.

Prognostic Index and Risk groups	Proportion of patients (%)	5-year overall survival (%)
IPI		
Low (0–1)	34	88
Low-intermediate (2)	23	76
High-intermediate (3)	23	67
High (4–5)	20	54
R-IPI		
Very good (0)	9	93
Good (1–2)	48	81
Poor (3–5)	43	61
Age-adjusted IPI (patients ≤60 years)		
Low (0)	22	83
Low-intermediate (1)	32	69
High-intermediate (2)	32	46
High (3)	14	32
NCCN-IPI		
Low (0–1)	13	92
Low-intermediate (2–3)	41	84
High-intermediate (4–5)	36	63
High (6–8)	10	49

Factors in IPI and R-IPI: Age >60 years, LDH > upper limit normal (ULN), Stage III or IV, ECOG performance status >1, no. of extranodal sites >1.
Factors in age-adjusted IPI: Stage III or IV, LDH > ULN, ECOG performance status >1.
Factors in NCCN-IPI: Age: >40 to ≤60 (1 point), >60 to ≤75 (2 points), >75 (3 points). LDH radio: >1 to ≤3 (1 point), >3 (2 points). Stage III or IV (1 point). ECOG performance status >1 (1 point). Extranodal disease: bone marrow, CNS, liver or GI tract, lung (1 point).

Staging

The Ann Arbor staging system, initially developed for Hodgkin lymphoma, is used in adults with B-cell NHL (see Chapter 25). Historically a staging bone marrow biopsy (BMB) was performed at baseline. The presence of high-grade disease in the bone marrow is prognostically important, and may direct the need for CNS prophylaxis or identify concomitant low-grade disease. However, most guidelines now suggest BMB can be omitted where baseline PET imaging has been performed. Radiography with computerised tomography (CT), magnetic resonance imaging (MRI) or PET scanning are used for initial staging of the disease and are of value in monitoring response to therapy and detection of residual disease or relapse. As treatment may differ for patients with stage I disease and more advanced stages, especially in the context of DLBCL, a PET scan at diagnosis can have significant therapeutic implications. At the end of therapy, PET has been incorporated into the revised response criteria.

Cerebrospinal fluid (CSF) examination including cytological assessment by cytospin and immunophenotyping should be considered if there are clinical signs of CNS disease, if there are radiological features suggesting CNS invasion or in cases exhibiting features associated with very high risk of CNS relapse.

Treatment

Treatment of aggressive B-cell NHL is mainly based on the histological findings, although therapeutic decisions are influenced by the patient's age, co-morbidities, performance status and disease extension. There are also a number of subtypes with distinct clinical and pathological features and therapeutic strategies that will be discussed separately. Often these lymphoma subtypes are potentially curable and thus access to timely sophisticated diagnostic services and expert clinical opinion is necessary to ensure accurate diagnosis and tailored therapy.

Particular considerations prior to therapy

A formal assessment of cardiac function is recommended for older patients and those with risk factors for cardiovascular disease prior to anthracycline therapy. Accurate analysis of renal function should be considered before the administration of high-dose methotrexate, platinum-containing regimens and high doses of other renally-excreted agents. Serology for HIV and hepatitis B (HBsAg, HBcAb and HBsAb) and hepatitis C should be performed in all patients as appropriate concomitant antiviral therapy may be indicated during and after therapy and can improve outcome and reduce morbidity of therapy.

Diffuse large B-cell lymphoma

The updated WHO classification from 2022 (Table 27.1) continues to recognise large B-cell lymphoma, not otherwise specified (DLBCL, NOS) as an entity in its own right. However, the principles of diagnosis, prognostication and management of DLBCL apply to several other subtypes including high-grade B-cell lymphoma (HGBL), and as a result, DLBCL is often used as an umbrella term for all related entities.

DLBCL is by far the most common NHL, representing approximately 30% of all newly diagnosed NHL and 85% of all aggressive B-cell lymphomas. According to the latest Haematological Malignancy Research Network's registry data, the overall annual incidence for DLBCL in the UK is 8.2 per 100,000 with a median age at diagnosis of 69.7 years and slight male predominance (1.2:1). Although DLBCL is potentially curable, it remains a challenging lymphoma to manage because of the biological and clinical heterogeneity of the disease and the poor outcomes in patients not responding to first-line treatment.

Aetiology

The majority of DLBCL cases have no underlying causative factor and arise *de novo*. However, a small proportion occurs as a result of transformation from a previously indolent disorder such as follicular lymphoma, marginal zone lymphoma or chronic lymphocytic leukaemia. Indeed, transformations of indolent B-cell lymphoma are now a separate entity within the WHO 2022 update. A small proportion of cases are associated with risk factors occurring in the setting of an underlying immunocompromised state including concomitant infections with EBV and HIV, post-solid organ transplantation and in elderly patients.

Pathogenesis and molecular basis of DLBCL

DLBCL is a clonal neoplasm of large B-lymphoid cells with a diffuse growth pattern. There is a growing appreciation of the marked biological heterogeneity of DLBCL, NOS through advances in technology leading to continued evolution of the classification of this disorder.

Cell-of-origin based typing

Pivotal gene expression profiling (GEP) studies in the early 2000s provided the first biological-based cell-of-origin (COO) classification reflecting the different stages of B-cell development from which the malignant B-cells arose. This approach identified two distinct binary groups: germinal-centre B-cell (GCB-DLBCL) and post-germinal centre-activated B-cell (ABC-DLBCL). Notably, about 10–15% of DLBCLs fell into an 'unclassified' COO subtype. The derivation of each subtype from differing COO explained their distinctive molecular features, clinical characteristics and prognostic information and thus the 2016 revision of the WHO classification recognised these two COO-based subtypes of DLBCL.

The GCB-DLBCL subtype expresses genes reminiscent of normal germinal-centre B-cells such as CD10 and LMO2. They are characterised by high expression of the transcriptional repressor gene, *BCL6*, which can also be translocated or mutated in DLBCL and exhibit highly mutated immunoglobulin genes with ongoing somatic hypermutation (SHM), further evidence that supports its germinal-centre origin. In contrast, ABC-DLBCL corresponds to post-GC B-cells characteristically expressing several genes that are normally upregulated in plasma cells but are blocked from terminal differentiation into plasma cells by additional genetic alterations that interfere with Blimp1, the master regulator of plasmacytic differentiation.

Genetically, ABC and GCB-DLBCLs are driven by their dependence on different oncogenic pathways. Chronic B-cell receptor (BCR) signalling and constitutive NF-κB activation are pathogenic hallmarks of ABC-DLBCLs, mediated through distinct genetic alterations in components of the *BCR* (*CD79A* and *CD79B*), *CARD11*, *MYD88* and *TNFAIP3*. On the other hand, GCB-DLBCLs primarily harbour genetic alterations in chromatin-modifying enzymes (*EZH2*, *CREBBP* and *KMT2D*). Additionally, approximately 40% of GCB-DLBCLs have a t(14;18) *BCL2* translocation that leads to constitutive overexpression of *BCL2* and over 50% of GCB-DLBCLs harbour *BCL2* mutations.

Importantly, COO-based stratification of DLBCL patients showed clinical prognostic relevance, with GCB-DLBCL demonstrating more favourable responses and overall survival (OS) rates compared with ABC-DLBCL, independent of the IPI, following treatment in the pre-rituximab era. Moreover, even in the setting of combination immunochemotherapy, the worse outcome for ABC-DLBCL was not abrogated (3-year progression-free survival (PFS): 74% GCB; 40% ABC; 3-year OS: approximately 80% GCB; 45% ABC, $P < 0.001$). These significant differences in outcomes inspired a number of clinical studies attempting to tailor treatment in accordance with the COO subtype, with particular emphasis to improve ABC-DLBCLs (see section *Treatment: First-line – advanced-stage disease*).

Molecular-based typing

The dawn of higher-resolution genomic technologies has brought about a more in-depth understanding of the underpinning molecular heterogeneity in DLBCL-NOS. Unlike the COO derived from GEP, these recent studies utilise broad genomic approaches including exome, targeted gene sequencing and copy number analyses to identify subtypes based on genetic alterations. With nearly 2000 primary DLBCL cases across three recent studies, a molecular classification-based approach was developed using the genetic attributes together with iterative

statistical modelling to identify subtypes based on the enrichment of driver mutations and specific biological pathways. Although, in each of these studies, the molecular classifiers were developed using different technologies, approaches and statistical methods and each with its own independent nomenclature, there was broad consensus across the studies lending weight to the robustness and validity of the molecular subtypes identified. Between five and seven molecular subtypes were described in each of these studies. The key molecular groups consistent across the three studies include:

1 MCD subtype (equivalent to C5 and MYD88 subtypes in the parallel studies): Characterised by mutations in *MYD88* L265P and *CD79B* leading to NF-κB activation and chronic BCR signalling and immune editing through mutations in *HLA A-C* genes. The majority of this subtype are ABC-DLBCL cases and frequently have extra-nodal site involvement including the testes, breast and CNS. Notably, PCNSL and primary testicular lymphomas have overlapping genetic profiles with this subtype.
2 EZB subtype (equivalent to C3 and BCL2): The majority of which are GCB-DLBCL cases characterised by *BCL2* translocations, frequent mutations in histone modifiers such as *CREBBP*, *EZH2* and *KMT2D*. Follicular and transformed follicular lymphoma share many genetic features with this subtype. A recent gene expression-based signature, molecular high grade (MHG) or double hit (DHITsig), that identifies a poor prognostic group of GCB-DLBCLs, is a subset within the EZB group, the majority of which expectedly harbour *MYC* aberrations.
3 BN2 subtype (equivalent to C1 and *NOTCH2*): Characterised by *BCL6* translocations and activating *NOTCH2* mutations. This subtype consists of DLBCLs that are mostly ABC-DLBCL or unclassified DLBCL. The genetic profile shares similarities to marginal zone lymphomas.
4 ST2 subtype (closely related to C4; *TET2/SGK1* and *SOCS1/SGK1*): Frequent truncating mutations in *SGK1* and *TET2* comprising predominantly of GCB-DLBCLs.

Additional groups identified but not aligned across the studies perhaps due to differences in methodology include:

- A53 subtype (equivalent to C2) comprised both ABC- and GCB-DLBCL cases and typified by widespread genomic instability including *TP53* mutations and multiple copy number alterations.
- N1 subtype was identified in only one study and is the rarest subtype occurring in less than 2% of DLBCLs, characterised by *NOTCH1* mutations and predominantly ABC-DLBCLs.

These genetic subtypes are linked to distinct clinical outcomes following R-CHOP therapy. The worst outcomes occur in the N1 (5-year OS 22%), EZB-MYC+ subset and *MCD/C5/MYD88* (approximate 5 years OS 40%) groups. Intermediate outcomes are seen in the BN2/C1/NOTCH2 and A53 groups (approx. 5 years OS 50%) whereas the most favourable outcomes are in patients within the ST2 and EZB-MYC- groups (approx. 5 years OS 60–65%). Altogether, these studies suggest a step-change beyond the existing binary COO framework to a modern molecular taxonomy classification system in DLBCL (Figure 27.1) with the potential for improved risk stratification and patient selection for novel therapies that can target distinct biological dependencies and vulnerabilities.

Role of the microenvironment in DLBCL

Less attention has been paid to the microenvironment in DLBCL although there is increasing evidence that a lymphoma-promoting niche and immune-modulation are relevant. Escape from immune surveillance is a common feature in DLBCL through loss of MHC class I and class II molecules; some of this is driven via genetic mechanisms including recurrent alterations in *EZH2*, *CREBBP* and *B2M*. Another immune escape mechanism is increased expression of PDL1/PDL2 on malignant cells occurring in a subset of DLBCLs. This might identify a cohort of patients that may respond to anti-PD1 therapy. More recently, transcriptomic analyses of the microenvironment of 4655 DLBCLs suggest that DLBCLs can be categorised into four major microenvironment groups each associated with characteristic biological features and clinical behaviour. This together with the molecular subtypes may facilitate alternative therapeutic approaches beyond the conventional R-CHOP.

Clinical prognostic factors

Despite advances in the treatment of DLBCL, the outcome of patients with DLBCL remains variable. Although potentially curable, about 40% of patients will die with relapsed or refractory disease. There is therefore a pressing need to identify patients for whom standard treatment measures are inadequate so that alternate therapeutic strategies can be employed. Clinical prognostic features remain surrogates for the underlying biological differences in patients; however, they continue to be the most widely used methods for delineating risk groups in clinical practice.

Table 27.2 summarises the most frequently used prognostic scoring systems in DLBCL. The International Prognostic Index (IPI) was devised in 1993 and remains the most widely used prognostic tool. It incorporates five clinical parameters determined at diagnosis, resulting in four risk groups with predicted 5-years OS ranging from 26 to 73%. An age-adjusted IPI was also defined for patients <60 years old and a revised IPI (R-IPI) outlined in the rituximab era with 3 risk groups identified. More recently, clinical data from the National Comprehensive Cancer Network (NCCN) database has allowed an 'enhanced IPI' tool to be devised. This tool accounted for the continuous nature of specific variables such as LDH as well as the specific prognostic impact of particular extra-nodal sites. Four risk groups were developed and compared favourably with the IPI, with the NCCN-IPI being better at discriminating low- and high-risk

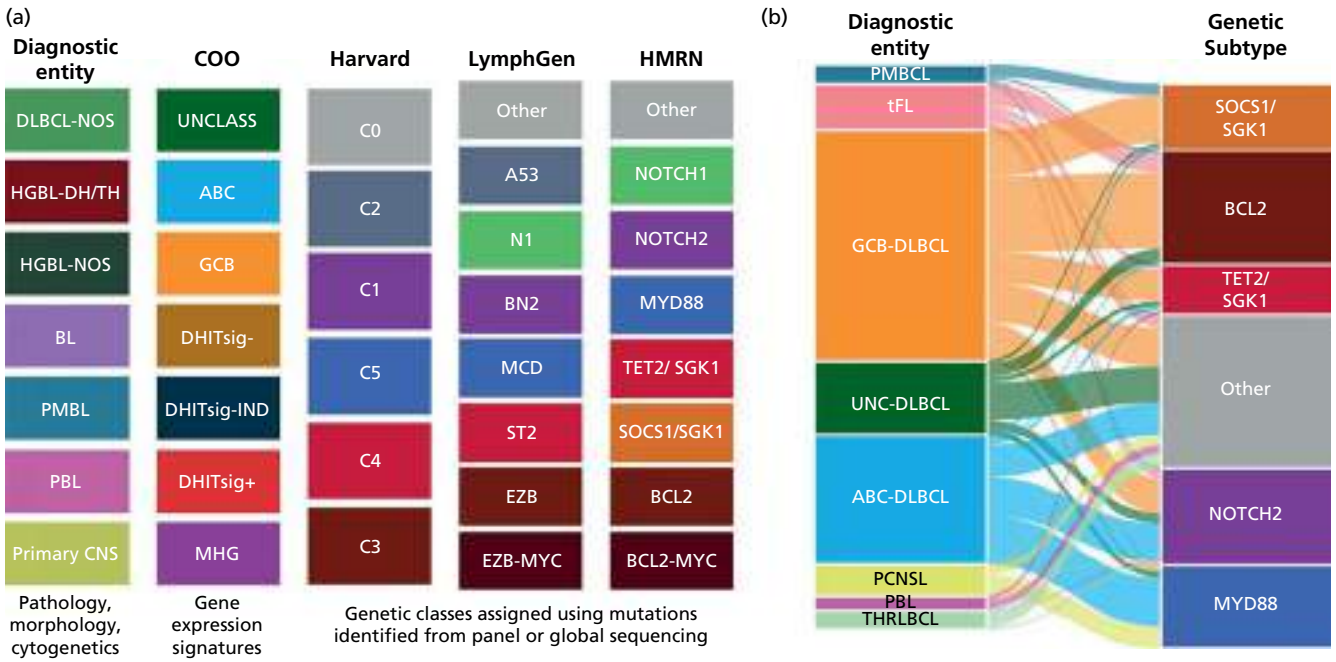

Figure 27.1 Evolution of the molecular classification DLBCL and related lymphoma entities. (a) Column 1 shows diagnostic entities closely related to DLBCL. Column 2 represents the subtypes defined by GEP. Columns 3–5 demonstrate the three recent studies (Harvard, LymphGen and HMRN) that have led to the molecular subtypes of DLBCL; (b) As an example, the HMRN classification is used to illustrate how the molecular subtypes relate to the diagnostic entities. (Source: Morin RD, Arthur SE, Hodson DJ (2021) Molecular profiling in diffuse large B-cell lymphoma: why so many types of subtypes? *B. J. Haem*., Open Access, https://doi.org/10.1111/bjh.17811/ British Society for Haematology and John Wiley & Sons Ltd/CC BY 4.0.).

subgroups compared to the IPI. Despite the validity of the various IPI risk models, they remain limited in their ability to predict which patients will follow a very aggressive course.

Treatment: first-line – limited-stage disease

Definitions of limited-stage disease vary, but it is commonly described as stage I or II disease that is anatomically localised, non-bulky (largest mass <7.5–10 cm) and without associated 'B' symptoms. Twenty-five to thirty percent of DLBCL present with limited-stage disease, and whilst the prognosis is excellent with modern therapy, a pattern of delayed relapse has been recognised.

Prior to the development of effective combination chemotherapy, radiotherapy was often employed as a single-modality treatment with some durable remissions. However, a significantly improved disease-free survival (DFS) and OS in limited-stage DLBCL patients who received combined modality treatment with brief chemotherapy course (three cycles of R-CHOP) followed by consolidative radiotherapy has been reported in a prospective randomised trial. The 5-year OS and PFS for stage I and II disease treated with R-CHOP and consolidative radiotherapy were 92 and 82%, respectively; whereas without radiotherapy, OS and PFS were 73% ($P = 0.0007$) and 68% ($P = 0.0003$), respectively. Until recently, abbreviated immunotherapy (three-to-four cycles of R-CHOP) followed by involved field radiotherapy (IFRT) has been widely accepted as the preferable approach for all early-stage DLBCL with non-bulky disease, especially at sites of presenting disease where radiation is well tolerated with lower risks of late toxicities. An alternative approach is following a full course of immunochemotherapy (six cycles of R-CHOP), omitting radiotherapy if the site of disease carries a risk of significant toxicity.

Recent trials have focussed on trying to minimise toxicity by identifying a subgroup of low-risk patients where abbreviated chemotherapy alone may be sufficient. The FLYER trial, published in 2019, showed that four cycles of R-CHOP plus two additional doses of rituximab was non-inferior to six cycles of R-CHOP in young patients with limited-stage, non-bulky DLBCL and age-adjusted IPI of 0. There was no OS difference between the groups and adverse events were reduced in the four-cycle group. Whether the two additional doses of rituximab confer any benefit is unclear, and it is crucial that this approach is restricted to patients meeting the strict criteria employed in the FLYER trial. Further trials are ongoing investigating whether using a PET-adapted approach can refine this approach further, with some initial results suggesting that early

PET response (after two cycles) can identify patients who benefit from abbreviated chemotherapy.

Primary extranodal DLBCLs have traditionally been included in many of the large studies focussing on early-stage disease; however, involvement with certain organs with DLBCL has distinct therapeutic implications when occurring as primary sites of disease.

Testicular

Testicular involvement by DLBCL is associated with a worse clinical outcome than many other subtypes of DLBCL, in particular with a high risk of CNS relapse. Management of this DLBCL subtype is often based on the protocol used in the phase II IELSG10 study which involved 6(-8) cycles of R-CHOP-21, four doses of intrathecal methotrexate and radiotherapy to the contralateral testis for all patients and to regional lymph nodes for stage II disease. Outcomes in the 53 patients enrolled were better than historical controls, with 5-year PFS and OS of 74 and 85% respectively, and cumulative incidence of CNS relapse of 6%.

On the basis of these results, and an increased recognition of the role of intravenous (IV) methotrexate in CNS prophylaxis, the subsequent IELSG30 trial had the same chemotherapy/radiotherapy protocol but incorporated two courses of IV methotrexate ($1.5\,g/m^2$) at the end of R-CHOP chemotherapy (in addition to four doses of intrathecal liposomal cytarabine). Recently presented data from this study (n = 54) reported no CNS relapses during the median 6 years of follow-up although late extranodal relapses were observed. A PFS of 88% at median follow-up of 5 years was reported.

Breast

DLBCL is the most common subtype of primary breast lymphoma. Data to inform treatment of breast DLBCL is largely retrospective, but several studies have reported high rates of relapse both in the ipsilateral or contralateral breast and in the CNS. As a result, CNS prophylaxis is often recommended as well as radiotherapy due to the local relapse risk. Six cycles of immunochemotherapy are usually delivered, with one retrospective study from the pre-rituximab era showing benefit for patients receiving more than three cycles.

Gastric

Patients with gastric DLBCL are generally treated with a similar approach to other sites of localised disease, either with six cycles of R-CHOP alone or three cycles of R-CHOP followed by radiotherapy. Surgery is generally avoided unless required for complications such as perforation, obstruction or bleeding. Some small studies have reported induction of remission with eradication of *Helicobacter pylori* (HP) alone in gastric DLBCL, both in cases with and without evidence of underling mucosa-associated lymphoid tissue (MALT) lymphoma. If this approach is followed, close monitoring for treatment failure is critical, with early institution of chemotherapy/radiotherapy if remission is not achieved.

Bone

Although bone involvement is seen reasonably frequently in advanced-stage DLBCL, lymphoma affecting bone as the only site of disease is relatively rare. Optimal therapy for primary bone DLBCL is not well-defined, guided mainly by retrospective studies either before or after the introduction of rituximab. The use of radiotherapy is controversial, with disagreement between studies regarding its potential benefit. Three cycles of R-CHOP with radiotherapy (in truly localised disease) or six cycles of R-CHOP with or without radiotherapy are the most frequently employed strategies for this entity.

Treatment: first-line – Advanced-stage disease

Patients with more advanced stages of disease (Stage 2B to Stage 4) should receive combination immunochemotherapy. Several studies have shown the benefit of adding Rituximab to chemotherapy making R-CHOP or R-CHOP-like regimens the gold standard in advanced-stage DLBCL. In recent years, several large phase III trials have attempted to improve on outcomes with R-CHOP, with a summary of selected key completed clinical trials in first-line DLBCL outlined in Table 27.3. Broadly speaking, these attempts have involved either intensification of immunochemotherapy, use of consolidative autologous stem cell transplant (ASCT) or, more recently, addition of a novel agent 'backbone' to (R)-CHOP. The positive trials will be discussed below. Approaches which have failed to show a benefit include intensification of R-CHOP given every 21 days to that given every 14 days; use of the infusional and dose-adjusted DA-EPOCH-R regimen (see Table 27.4); replacement of rituximab with obinutuzumab; addition of ibrutinib to R-CHOP in non-GC subtype of DLBCL; maintenance rituximab following R-CHOP; consolidative autologous stem cell transplant following R-CHOP and incorportation of lenalidomide maintenance in older patients with DLCBL following R-CHOP induction.

Intensified chemotherapy approaches

The only regimen to date conferring an overall survival benefit over R-CHOP in a randomised study has been dose-intensive R-ACVBP (see Table 27.4) followed by sequential consolidation (rituximab, ifosfamide, etoposide and cytarabine). The LNH03-2B trial, published in 2011, demonstrated significantly improved survival with this regimen in patients aged <60 years with low-intermediate risk compared to standard R-CHOP (3 years PFS 87 versus 73%, HR 0.48, p = 0.0015; 3 years OS 92 versus 84%, p = 0.0071). However, significant toxicities, in particular haematological, have limited its application in the real-world setting, especially in older patients.

Table 27.3 Selected key phase III trials in first-line DLBCL.

Study	Regimen	No. of patients	Criteria	Age	Outcome
MInT	**A.** R-CHOP-like x6 Versus **B.** CHOP-like x6	824	aaIPI: 0 or 1	18–60 years	3 years EFS: 79% (A) versus 59% (B)* 3 years OS: 93% (A) versus 84% (B)*
LNH03-2B (GELA study group)	**A.** R-ACVBP x4 and consolidation Versus **B.** R-CHOP x8	380	aaIPI: 1	18–59 years	3 years EFS: 81% (A) versus 67% (B)* 3 years PFS: 87% (A) versus 73% (B)* 3 years OS: 92% (A) versus 84% (B)*
NCRI	**A.** R-CHOP-14 x6 Versus **B.** R-CHOP-21 x8	1080	All IPIs	>18 years	2 years PFS: 75.4% (A) versus 74.8% (B)# 2 years OS: 82.7% (A) versus 80.8% (B)#
RICOVER-60 (German High-Grade Non-Hodgkin's lymphoma study group)	**A.** CHOP-14 x6 Versus **B.** CHOP-14 x8 Versus **C.** R-CHOP-14 x6 Versus **D.** R-CHOP-14 x8	1222		61–80 years	3 years EFS: 47.2% (A) versus 53% (B) versus 66.5% (C) versus 63.1% (D) 3 years OS: 67.7% (A) versus 66% (B) versus 78.1% (C) versus 72.5% (D)
LNH03-6B (GELA study group)	**A.** R-CHOP-14 x8 Versus **B.** R-CHOP-21 x8	602	aaIPI ≥1	60–80 years	3 years EFS: 56% (A) versus 60% (B)#
SWOG-9704 (US and Canadian Intergroup)	**A.** CHOP or R-CHOP x6 followed by ASCT Versus **B.** CHOP or R-CHOP x8	370 (248 DLBCL)	aaIPI: 2 (high-intermediate) or 3 (high risk)	15–65 years	2 years PFS: 69% (A) versus 55% (B)* 2 years OS: 74% (A) versus 71% (B)#
Alliance/CALBG 50303	**A.** DA-EPOCH-R x 6 Versus **B.** R-CHOP x 6	491	All IPIs	≥18 years	2 years PFS: 79% (A) versus 76% (B)# 2 years OS: 87% (A) versus 86% (B)#
GOYA	**A.** Obinatuzumab-CHOP Versus **B.** R-CHOP	1418	aaIPI ≥2 or IPI = 1 and age ≤60 years or IPI = 0 and bulk (≥7.5 cm)	≥18 years	3 years PFS: 70% (A) versus 67% (B)# 3 years OS: 81% (A) versus 81% (B)#
REMoDL-B	**A.** R-CHOP + Bortezomib Versus **B.** R-CHOP	918	Stage I and bulk (>10 cm) – IV, All IPIs, sufficient material for GEP	≥18 years	30 m PFS: 74% (A) versus 70% (B)# 30 m OS: 83% (A) versus 82% (B)#
PHOENIX	**A.** R-CHOP + Ibrutinib Versus **B.** R-CHOP	838	Stage II–IV, R-IPI ≥1, non-GCB by Hans IHC algorithm	≥18 years	3 years PFS: 70% (A) versus 67% (B)# 3yr OS: 83% (A) versus 81% (B)#

Table 27.3 (Continued)

Study	Regimen	No. of patients	Criteria	Age	Outcome
ROBUST	**A.** R-CHOP + Lenalidomide Versus **B.** R-CHOP	570	Stage II–IV, IPI ≥2, ABC subtype by GEP Nanostring	≥18 years	2 years PFS 67% (A) versus 64% (B)[#] 2 years OS 79% (A) versus 80% (B)[#]
POLARIX	**A.** R-CHP + Polatuzumab vedotin Versus **B.** R-CHOP	879	Stage I–IV, IPI ≥2	18–80 years	2 years PFS: 77% (A) versus 70% (B)* 2 years OS: 89% (A) versus 89% (B)[#]

* Statistically significant difference ($P < 0.05$).
[#] Not statistically significant.
aaIPI, age-adjusted IPI; CALBG, Cancer and Leukaemia Group B; EFS, event-free survival; GELA, Groupe d'Etudes des Lymphomes de l'Adulte; NCRI, National Cancer Research Institute; OS, overall survival; PFS, progression-free survival; SWOG, Southwest Oncology Group.

Table 27.4 Chemotherapy protocols used in high-grade NHL.

ACVBP	Doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisolone
BEAM/ mini-BEAM	BCNU, etoposide, cytarabine, melphalan
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisolone
CODOX–M/ IVAC	Cyclophosphamide, doxorubicin, high-dose methotrexate/ifosfamide, etoposide and high-dose cytarabine
DHAP	Dexamethasone, high-dose cytarabine and cisplatin
DVIP	Dexamethasone, etoposide, ifosfamide and cisplatin
EPOCH/ DA-EPOCH	Etoposide, prednisolone, vincristine, cyclophosphamide, doxorubicin
ESHAP	Etoposide, cytarabine, methylprednisolone, cisplatin
GCVP	Gemcitabine, cyclophosphamide, vincristine, prednisolone
GemOx	Gemcitabine, oxaliplatin
Hyper CVAD	Hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone
ICE	Ifosfamide, carboplatin, and etoposide

Based on results from the MiNT study, conducted prior to the use of PET scans in response assessment, R-CHOP with the addition of radiotherapy to sites of primary bulky disease was previously recommended for young patients with low or intermediate risk IPI. In recent years, the value of consolidative radiotherapy has been questioned, with large retrospective datasets and sub-analyses from prospective trials demonstrating patients with a complete metabolic response (CMR) on post-treatment PET have favourable outcomes without the use of radiation therapy.

For patients with a high-risk IPI score of 3–5 for which the PFS of <50% at 3 years following R-CHOP is unsatisfactory, there remains no consensus on the appropriate treatment strategy. In the UK, a single arm National Cancer Research Institute (NCRI) phase II study explored the feasibility of using the intensified R-CODOX-M/R-IVAC regimen (see Table 27.4) in patients <65 years with high risk DLBCL (IPI 3–5). Outcomes in this high-risk cohort (9% CNS involvement and 53% performance status (PS) ≥2) were promising with 3-year PFS and OS 68.4 and 76.2% respectively. However, toxicity was significant with treatment-related mortality (TRM) of 4.3%, highest in patients >50 years and PS≥2. These results require confirmation in a randomised study before its use can be adopted widely; however, it remains an option for selected high-risk patients deemed fit enough for such an intensive approach.

Addition of novel agents

An increased understanding of the molecular heterogeneity of DLBCL has provided the basis for pre-clinical studies identifying subtypes which may benefit from the addition of targeted novel agents. Several phase 3 trials have now been performed using the 'R-CHOP + *X* versus R-CHOP' design (see Table 27.3). The first of these trials, reported in 2019, investigated the addition of Bortezomib, a proteasome inhibitor which causes inhibition of the NF-κB pathway and has activity thought to potentially benefit patients with ABC subtype DLBCL. GEP at diagnosis was used to classify COO, and this information was combined with IPI classification to allow stratified randomisation between R-CHOP alone and R-CHOP + Bortezomib from cycle 2 onwards. The initial report did not suggest a survival improvement although on longer follow-up this was seen for

patients with the activated B-cell subtype and so-called molecular high-grade group. The lack of availability and standardisation of GEP has limited use of this approach.

The phase III POLARIX trial has recently demonstrated superior PFS with substitution of vincristine with the antibody-drug conjugate Polatuzumab vedotin (pola-R-CHP) for patients with intermediate- or high-risk DLBCL. Patients in the pola-R-CHP arm had 2 year PFS of 76.7% compared to 70.2% in the R-CHOP arm, with fewer patients (22.5 versus 30.3%) requiring subsequent anti-lymphoma therapy. However, there was no OS difference between the two arms (2 year OS 88.7 versus 88.6%), possibly reflecting the efficacy of novel therapies in the second-line setting and beyond such as CAR T-cells. There was no stratification based on COO or molecular subtyping, although interestingly post-hoc subgroup analyses suggested a possible benefit for patients with ABC-subtype DLBCL. The biological rationale for this is unclear. As a result of these findings, Pola-R-CHP has been approved in the USA and Europe, however the additional cost of Polatuzumab vedotin and the lack of OS benefit seen may hinder its widespread application.

Special considerations – DLBCL in the elderly

Approximately 50% of DLBCL diagnoses occur in patients older than 60 years with this number expected to rise alongside the increasing age of the population. Patients older than 60 years represent a heterogeneous population with varying levels of general health, co-existing co-morbidities and an increased susceptibility to treatment toxicities. A comprehensive geriatric assessment is increasingly performed prior to treatment decisions to ensure all these factors are considered. With key randomised trials in DLBCL frequently excluding patients >80 years, this patient group can be very challenging to manage with no firmly established standard of care.

Results of the GELA study (LNH98-5) demonstrated that the addition of rituximab to CHOP-21 improves PFS and OS in patients aged 60–80 years. As a result, R-CHOP-21 remains the standard therapy for fit patients under the age of 80 without cardiac dysfunction, whilst omission of anthracycline is commonly considered in those that have proven cardiac dysfunction. For this latter cohort of older patients with cardiac co-morbidities, a phase II study has demonstrated efficacy using a regimen where anthracycline is substituted with gemcitabine, R-GCVP, with an ORR of 61% and 2-year PFS and OS of 49.8 and 55.8% respectively. Alternative options in this setting include substituting doxorubicin with etoposide (R-CEOP) or rituximab, gemcitabine and oxaliplatin (R-GemOx).

For patients older than 80 years without significant cardiac dysfunction, a phase II trial from the LYSA group demonstrated that dose reduction of R-CHOP – so-called R-miniCHOP – is deliverable for very elderly patients with 4-year DFS of 57.9% and a clear plateau in survival curves. Of note, the TRM rate was 8%, highlighting the need for judicious supportive care and attention to dose-reductions in this group. Subsequent trials have investigated the use of pre-phase therapy with vincristine and prednisolone, with results demonstrating a significant improvement in performance status prior to definitive chemotherapy, and reduction in toxicity including neutropenia and neutropenic fever. Routine G-CSF prophylaxis is often recommended for all patients over 65 years old in a further attempt to reduce risk of infective toxicity.

A systematic review of 13 retrospective studies and 5188 patients published in 2021 demonstrated that higher R-CHOP dose intensity was associated with improved survival in those aged <80 years, but subgroup analyses of patients aged ≥80 years showed that survival was not affected by reduced dose intensity. Acknowledging the limitation of mainly retrospective studies informing this analysis, it supports the broadly accepted approach of attempting full dose R-CHOP in those aged <80 years and reserving R-miniCHOP for those aged ≥80 years or with significant co-morbidity.

CNS prophylaxis

Systemic disease progression is the primary cause of treatment failure in DLBCL; however, relapse within the CNS occurs in approximately 5% of patients. The outcome of those who develop CNS relapse is extremely poor, with a median survival of less than 6 months. However, prophylactic treatment aimed at preventing this devastating outcome remains a very controversial area with little robust evidence to guide practice.

The definition of 'high-risk' for CNS relapse has been modified over the years, with the most widely adopted model for patient selection being the 'CNS-IPI', developed by the German High-Grade Lymphoma Study Group. Univariable and multivariable analyses of a training cohort of 2164 patients from prospective trials were performed with validation in a large registry cohort, resulting in a model consisting of the established IPI factors plus involvement of kidney and/or adrenal glands (see Table 27.5). Patients with a high CNS-IPI score (four to six points) are frequently offered CNS prophylaxis, but this remains an imperfect approach given that ~90% of patients in this group will receive prophylaxis unnecessarily.

Other risk factors warranting consideration for CNS prophylaxis, independent of the CNS-IPI, are the presence of three or more extranodal sites or particular extranodal site involvement including testicular, renal, adrenal, breast and uterine. Previous data suggested that ABC-subtype DLBCL (established by GEP) or 'double hit' HGBCL also conferred an increased risk of CNS relapse. The new molecular classification of DLBCL may also allow more precise identification of subgroups with high CNS risk. Another area of interest is the use of circulating tumour DNA (ctDNA) analysis in CSF at diagnosis to detect occult CNS disease with much higher sensitivity than traditional CSF analysis by flow cytometry.

Intrathecal (IT) chemotherapy was previously considered the CNS prophylaxis of choice for selected patients, but there is now

Table 27.5 Central nervous system international prognostic index.

CNS-IPI risk group	2-year rates of CNS relapse (%)	Proportion of patients: training cohort (%)	Proportion of patients: validation cohort (%)
Low (0–1 points)	0.6	46	31
Intermediate (2–3 points)	3.4	41	46
High (4–6 points)	10.2	12	23

CNS-IPI risk categories with corresponding 2 year rates of CNS relapse and proportion of patients in each category from the training (clinical trial patients) and validation (BCCA registry) cohorts (Schmitz *et al.* 2016). 1 point is scored for any of the following: age >60 years, LDH >normal, ECOG performance status >1, stage III/IV disease, extranodal involvement ≥2 sites, kidney and/or adrenal involvement.

cumulative evidence to suggest it has limited efficacy and therefore is generally not recommended in isolation. Intravenous high-dose methotrexate (HD-MTX) with folinic acid rescue has instead been widely adopted as CNS prophylaxis despite a lack of prospective data supporting its use and the risk of toxicity and interruptions to systemic therapy. Recently, two large retrospective analyses have addressed this topic. The first analysed 1384 patients receiving HD-MTX CNS prophylaxis either 'intercalated' between cycles or delivered at end of R-CHOP therapy. There was no difference in CNS relapse rate between the two approaches, with intercalated delivery introducing significant risk of R-CHOP delay, and the overall rates of relapse seen in high-risk patients were similar to published cohorts receiving no CNS prophylaxis. The second analysis of 2300 patients deemed at high risk of CNS relapse found no reduction in risk between patients who did (n = 410) and did not (n = 1890) receive HD-MTX. Taken together, these analyses suggest that HD-MTX is unlikely to significantly reduce rates of CNS relapse for the majority of patients deemed at high risk by traditional criteria, and if it is used it should be deferred until end of systemic therapy to avoid treatment delays.

In summary, current methods for identifying high-risk patients and the agents used for prophylaxis are suboptimal. Future studies are likely to focus on using modern diagnostic technology to identify 'molecular high-risk' patients, with incorporation of novel agents capable of penetrating the CNS and thus preventing the devastating consequences of CNS relapse.

Role of interim PET/CT in response assessment

Unlike in Hodgkin lymphoma where the prognostic utility of interim PET-CT is established, its role in DLBCL remains debatable. Initial studies using PET-CT for response assessment after two, three or four cycles of immunochemotherapy suggested an association between the result of the interim scan and patient outcome. However some studies have demonstrated interim PET-CT assessment in DLBCL to have low prognostic and positive predictive value with a high number of false positive interim scans confirmed by a biopsy of the FDG-positive site. What is evident in comparing these studies is a current lack of standardisation for the appropriate time point for interim response assessment (two, three or four) and the response criteria used, as different grading systems from visual assessment scales (such as the Deauville five-point scale) to quantitative measures (such as change in standardised uptake value; SUV) are employed and subject to wide inter- and intra-observer variability.

The most robust trial to date in this area is the PETAL study which enrolled 862 patients with aggressive B or T-cell lymphomas and included an interim PET-CT after two cycles of (R)-CHOP. PET-CT response was defined according to reduction in SUV. A positive interim PET-CT was associated with poor EFS (<50%) which was independent of IPI risk group. Crucially, however, patients with a positive interim PET-CT who were randomised to receive an intensified 'Burkitt-like' therapy instead of (R)-CHOP had no improvement in survival outcomes but did experience increased toxicity.

Greater harmonisation of interim PET-CT interpretation is required before this response modality can be transitioned to the standard of care. Studies are ongoing combining ctDNA assessment with interim PET-CT imaging to better identify true high-risk patients. However, there must be a prospectively evaluated strategy to improve outcomes for these patients before these assessments can be recommended in routine clinical practice.

Treatment: relapsed or refractory DLBCL

Up to 40% of patients do not respond to first-line therapy and fall into one of three categories: those relapsing after achieving CR, those achieving PR with residual disease or refractory patients. The majority of the progressions occur within the first 18 months, with rare relapses occurring more than 5 years after diagnosis. Once a patient inadequately responds to initial chemotherapy, the prognosis is poor with historical data reporting long-term OS of less than 10%. However, the treatment landscape for such patients has been transformed in recent years by the development of novel treatments, in particular chimeric antigen receptor (CAR) T-cell and bi-specific T-cell engager (BiTE) therapies.

High-dose chemotherapy and ASCT

Until recently, second-line chemoimmunotherapy followed by ASCT was recommended for patients considered fit enough as established from the results of the landmark prospective PARMA trial published in 1995, which demonstrated the superiority of intensified treatment in patients with chemo-sensitive relapse. PET-CT following second-line therapy and prior to ASCT is highly prognostic, with excellent outcomes seen in

patients with Deauville score 1–3 pre-ASCT. However, a CMR is not necessarily required to proceed to ASCT, as demonstrated in a recent retrospective analysis of 249 patients undergoing ASCT with a PET-positive partial response, where the 5-year PFS was reported at approximately 40%.

The second-line chemotherapy of choice, however, remains a subject of debate. The majority of favoured regimens include rituximab with either one or both of a platinum compound or ifosfamide. A prospective phase III trial showed there was no difference in survival between second-line regimens R-ICE (rituximab, Ifosfamide, carboplatin and etoposide) and R-DHAP (rituximab, dexamethasone, high-dose cytarabine and cisplatin). Another randomised prospective study comparing R-GDP to R-DHAP demonstrated similar response rates and survival between regimens but increased toxicity with R-DHAP.

Eligibility for high-dose therapy and ASCT is generally determined by medical fitness, comorbidities, performance status and patient preference. Age is often considered an important factor, with patients over 70 years old often not proceeding to ASCT unless they are exceptionally fit. A recent retrospective analysis demonstrated that toxicity of ASCT, in particular cardiovascular, was increased in a cohort of patients >70 compared to those aged 60–69. However, non-relapse mortality was comparable across the two groups and therefore age alone should not be considered a reason to withhold this therapy in selected patients.

There is no clear consensus regarding management of patients where ASCT is deemed unsuitable, either due to patient fitness or due to lack of response to second-line chemotherapy. Until recently, options for patients not deemed fit for ASCT were limited to less intensive chemotherapy regimens such as R-GemOx, palliative oral chemotherapy combinations, steroid monotherapy as palliation or best supportive care alone. However, with increased recognition of the more favourable toxicity profiles of novel cellular therapies and targeted agents (see below), less fit patients may also be considered for these approaches subject to local availability and thus focus on appropriate sequencing of therapies has become increasingly important.

CAR T-cell therapy

CAR T-cells are genetically modified T cells formed by combining the antigen-binding site of an antibody with the intracellular domain of a T-cell activation receptor. The first approved products for treatment of B-cell lymphomas involve autologous T-cells targeting CD19; however, several trials are ongoing investigating the use of other targets (including CD20 and CD22) and allogenic 'off-the-shelf' products. Initial approvals in most countries recommended consideration of CAR T-cells after failure of second-line therapy; however recent results of some of the phase III trials comparing CAR T-cells to chemotherapy and ASCT have led to approval of CAR T-cells for patients with disease which is refractory to R-CHOP or relapsing within 1 year.

Manufacture of CAR T-cells is complex and expensive, involving leukapheresis to obtain autologous T lymphocytes which are genetically modified (using viral vectors) with a gene encoding a CAR designed to direct the T cells against lymphoma cells. This modification occurs *ex vivo*, with subsequent expansion in a centralised production facility, before being transported back to the patient and infused. Managing patients in this period while the product is being manufactured can be challenging due to the aggressive nature of relapsed/refractory DLBCL, and some form of 'bridging' therapy (either corticosteroids, immunochemotherapy, radiotherapy or a combination of the above) may be required.

The therapy itself can also be associated with significant side effects, including neurological toxicity (immune effector cell-associated neurotoxicity syndrome, ICANS) and cytokine release syndrome (CRS). CRS is a severe systemic response to the activation and proliferation of CAR T-cells and is observed in a variable proportion of patients, depending on the product, but typically is transient and responds well to treatment including corticosteroids and tocilizumab (a monoclonal antibody against interleukin-6 receptor). ICANS is a neuropsychiatric syndrome that is usually seen in the context of CRS and can result in seizures and diffuse cerebral oedema in the most severe cases. Management is supportive, consisting primarily of corticosteroids and anti-epileptic medications.

There are currently three commercially available anti-CD19 CAR-T products – axicabtagene ciloleucel, tisagenlecleucel and lisocabtagene maraucel. They have all been FDA-approved following pivotal phase II trials but at present none have been compared directly in a randomised study. The three products have subtle differences in molecular structure, manufacturing processes and toxicity profiles. However, the efficacy appears to be broadly similar between products, with overall and complete responses reported in the range of 50–82% and 40–54% respectively. The longevity of these responses is less clear, although updated follow-up from the phase II study of axicabtagene ciloleucel has demonstrated that 44% of patients were alive at 4 years.

Despite the significant toxicities associated with CAR T-cell therapy, there is increased recognition that these are manageable and reversible for the majority, and overall the process is likely to be better tolerated than high-dose salvage chemotherapy and ASCT. As a result, patients previously deemed unfit for further treatment due to ineligibility for ASCT can now be considered for CAR T-cell therapy. The challenges going forward include reducing the manufacturing time of the products, investigating 'off-the- shelf' products which can be infused rapidly, reducing toxicities, identifying the optimal timing for such an approach and reviewing the response to other treatment modalities if CAR-T is ineffective. Finally, a major consideration is the socioeconomic considerations of such an expensive therapy, which may not be feasible in many healthcare settings with more limited resources.

Bispecific T-cell engagers (BiTEs) and bispecific antibodies

BiTEs are a new class of immunotherapy which conceptually function as linkers between T-cells and specific target antigens in an MHC-subtype independent manner. They consist of a protein fragment containing two separate single-chain variable regions. One end recognises CD3, expressed on all T-cells, while the other end recognises the target tumour antigen. The result is induction of cytotoxic T-cell-mediated tumour eradication. Bispecific antibodies are conceptually similar, but in this case, a whole antibody is engineered to have Fab portions which recognise two separate antigens, most commonly CD3 and CD20.

Following impressive results in patients with acute lymphoblastic leukaemia, a number of BiTE and bispecific antibody products have now been investigated for management of relapsed/refractory DLBCL. Initial BiTE products, e.g. blinatumomab, were cumbersome to deliver (continuous infusion) and associated with significant neurotoxicity. Several bispecific antibodies targeting CD3 and CD20 have now been developed which have longer half-lives, allowing delivery every 3–4 weeks, with subcutaneous administration possible for some. Although these therapies are associated with toxicities similar to those seen with CAR T-cells, namely CRS and ICANS, the rates of grade 3 and 4 toxicities are significantly lower than in CAR T-cells. Both glofitamab and epcoritamab are licensed in Europe for use in the third line plus treatment setting for DLBCL. Future trials directly comparing bispecific antibodies to CAR T-cell therapy, and earlier incorporation of these agents in the DLBCL treatment algorithm, are awaited with great interest.

Other novel therapies in relapsed/refractory DLBCL

One regimen being used increasingly in patients deemed unsuitable for ASCT is the combination of rituximab, bendamustine and polatuzumab vedotin (R-BP). Polatuzumab is an antibody-drug conjugate (ADC) targeting the B-cell antigen, CD79B. When compared to rituximab-bendamustine in a phase II trial of 80 patients, R-BP demonstrated significantly improved response rates and OS. Bendamustine does result in significant and often prolonged lymphopenia so consideration regarding appropriate timing of its administration is imperative. Loncastuximab tesirine is another ADC which instead targets CD19 and has demonstrated impressive response rates in a phase 2 trial, including in patients treated with prior CAR T-cell therapy.

Tafasitamab is humanised anti-CD19 monoclonal antibody, which demonstrated efficacy when combined with the immunomodulatory agent Lenalidomide in a phase 2 trial. Selinexor, a selective inhibitor of the nuclear export protein XPO1, has shown modest activity as a single agent for relapsed/refractory DLBCL, but is hindered by a significant toxicity profile. Other agents including venetoclax (BCL2 inhibitor), ibrutinib and tazemetostat (EZH2 inhibitor) have demonstrated limited efficacy as single agents but trials are ongoing investigating their use in combination therapies.

Despite showing efficacy in Hodgkin lymphoma, and in primary mediastinal B-cell lymphoma, programmed cell death protein 1 (PD-1) inhibitors have thus far failed to show significant benefit in DLBCL.

DLBCL in HIV-positive patients

People living with HIV (PLWH) have an increased risk of developing DLBCL, the second most common tumour in individuals with HIV. The development of DLBCL is related to older age, low CD4 cell count and no prior treatment with combination anti-retroviral therapy (ART). Patients tend to present with advanced-clinical stage, B symptoms and extra-nodal involvement. Factors associated with survival in the post-ART era are the IPI score and in some studies, a low CD4 cell count, but overall outcomes for PLWH have recently been demonstrated to be similar to HIV-negative counterparts with equivalent age-adjusted IPI.

Use of ART

The introduction of ART has led to better control of HIV viral replication and improved immune function. Previously, opinions differed as to whether ART should be continued during chemotherapy or not, with some concerns regarding potential adverse pharmacokinetic and pharmacodynamic interactions with chemotherapy and the potential for increased toxicity. However, with modern ART agents, these concerns regarding toxicity have not been realised, and there is now general acceptance that ART should be continued or initiated wherever possible. A combined approach to care involving HIV physicians and haemato-oncologists is essential, particularly when considering switching ARV agents to avoid chemotherapy interactions.

Choice of immunochemotherapy

There is much debate on whether R-CHOP or infusional regimens, such as DA-EPOCH-R, are most efficacious as first-line treatment in HIV-positive patients. However, there has been no randomised study comparing these regimens specifically in PLWH. A pooled analysis of US studies suggested that DA-EPOCH-R resulted in superior response rates and survival compared to R-CHOP, but the former regimen was used during a later time period (2002–2006) than the R-CHOP study (1998–2002), suggesting other variables, including supportive care and antiretroviral drug options, may have differed.

The role of rituximab in HIV-associated DLBCL has been controversial ever since a randomised phase III study of CHOP ($n = 50$) versus R-CHOP ($n = 99$), conducted in the US was published. Although there was a trend to improved response rate

with rituximab, (58 versus 47%, $p = 0.15$), a significant reduction in progression of lymphoma on treatment, and in death due to lymphoma, an increased death rate from infectious complications, particularly (9/15) in those with a CD4 count below 50 cells/mm^3, was observed. However, maintenance rituximab was included every 3 months in those who responded to R-CHOP. Subsequent phase II studies of R-CHOP (without maintenance rituximab) from Europe did not show an increased risk of infectious deaths, instead showing that rituximab was beneficial. A meta-analysis of prospective studies has confirmed the benefit in response rate and OS of the addition of rituximab to chemotherapy. Thus, the addition of rituximab to chemotherapy is now recommended for all patients with DLBCL with appropriate anti-microbial prophylaxis, pre-emptive G-CSF and prompt treatment of opportunistic infection.

Preventing CNS relapse

Historical data suggested high rates of both CNS involvement at baseline and of CNS relapse during or shortly after treatment for HIV-associated DLBCL. However, earlier estimates included patients with HIV-associated BL where CNS involvement is much more prevalent. Furthermore, it is unlikely that HIV infection itself is an independent risk factor for CNS relapse in DLBCL, but patients tend to present with other features which are known to increase the risk (*see section on* 'CNS prophylaxis' *above*). As a result, many guidelines now recommend applying the same criteria for PLWH as non-HIV-associated DLBCL when making decisions about CNS prophylaxis.

Relapsed/refractory disease

Treatment of refractory or relapsed DLBCL in the pre-ART era was disappointing with few clinically useful responses. Two large, retrospective, multi-centre studies performed by the EBMT have confirmed the feasibility and efficacy of HDT and ASCT for PLWH who respond to second-line chemotherapy. Thus, in the ART era, PLWH with chemosensitive relapsed DLBCL should be considered for ASCT according to the same criteria adopted for HIV-negative patients.

The pivotal trials of anti-CD19-directed CAR T-cell therapy in DLBCL excluded HIV-positive patients, and as a result, there was initial hesitancy in using this therapy for PLWH. However, there is now growing evidence suggesting the products can be manufactured adequately with comparable safety and efficacy to HIV-negative patients, and as a result, this can be considered in line with approvals for HIV-negative DLBCL.

Allogeneic stem cell transplantation in DLBCL

With the advent of CAR T-cells and other novel therapies, the role of allogeneic stem cell transplant in DLBCL is diminishing. There is no certainty regarding the existence of a strong graft-versus-lymphoma (GvL) effect in aggressive NHLs, whereas mortality following transplantation remains high. Traditionally, allogeneic transplant was considered for patients with chemosensitive disease who fail to adequately mobilise autologous stem cells or who relapse after ASCT. However, such patients will now be offered CAR T-cell therapy or other novel agents in countries where these are available, although allogeneic transplant remains a potential option where these treatments have failed or are not accessible. The choice of conditioning regimen employed should be individualised, based on balancing the risks of toxicity with a myeloablative approach versus the greater risk of relapse with reduced-intensity conditioning.

High-grade B-cell lymphoma with MYC and *BCL2* rearrangements (Double-hit lymphoma)

The previous WHO classification of haematopoietic and lymphoid tumours from 2008 included a borderline category named 'B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma', often referred to informally as 'high-grade lymphoma unclassifiable' or HGLUC. This category was used to describe high-grade B-cell malignancies that had intermediate cytological features between DLBCL and BL. Many cases in this category were found to be so-called 'double hit lymphoma (DHL)', carrying translocations of *MYC* and either *BCL2* or *BCL6*, or, more rarely, 'triple hit lymphoma (THL)', involving *MYC*, *BCL2* and *BCL6* translocations. The majority of DHL or THL are GCB-DLBCLs with genetic profiles similar to the molecular EZB subtype except *MYC-BCL6* DHLs, which are mostly ABC-DLBCLs. Clinical studies indicated that many DHLs were clinically aggressive with associated poor outcomes with standard DLBCL therapy (R-CHOP). Moreover, there was recognition that DHLs were morphologically heterogeneous, with some being diagnosed as DLBCL and some as HGLUC. As a result, the 2016 revision of the WHO classification created a unifying category, 'High-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements'. In the 2022 update, this has been modified to only include *MYC* and *BCL2* rearrangements. Due to the distinct characteristics of lymphomas with MYC and BCL6 rearrangements (without *Bcl2* rearrangements), this would now be placed under 'DLBCL, NOS' or 'HGBCL, NOS' depending on the cellular morphology. Importantly, this category includes only those cases with *MYC*/*BCL2* translocations identified by FISH – cases with overexpression of *MYC* and *BCL2* by immunohistochemistry are known as 'double-expressors'. Double-expressors account for around 25% of DLBCL cases and have been shown to have an inferior outcome with R-CHOP, but at present, this is not a specific diagnostic category in itself and treatment is as per non-double expressor DLBCL.

Incidence and clinical presentation

Initial estimates of both frequency and outcomes of DHL were skewed due to non-universal application of FISH testing, with early studies often only analysing cases exhibiting high-risk features. However, a large analysis from the Lunenburg Biomarker Consortium has provided the most robust estimates for DHL incidence, as well as providing insight into the heterogeneity of this diagnostic category. In over 2,000 DLBCL cases with biopsy material available for FISH analysis, *MYC* translocations were identified in 11% of patients. Of these cases, 53% had a concomitant rearrangement of *BCL2* and/or *BCL6*. Outcomes for *BCL2*-DHL, *BCL6*-DHL and THL were similar with all having inferior PFS and OS compared with those patients without translocations. Interestingly, this adverse prognostic impact was only observed in patients with DHL/THL cases in which *MYC* was translocated with an immunoglobulin chain (IG) partner.

Patients with DHL/THL frequently present with high-risk clinical features, including advanced stage, B symptoms, raised LDH and multiple extranodal site involvement. CNS involvement at diagnosis has been reported as frequently as 10%, although such figures are likely to be inflated by lack of universal FISH testing. Conversely, there are patients with DHL who present without high-risk features – such cases are likely to be underreported, and may represent a group who have similar outcomes with R-CHOP to non-DHL DLBCL.

Treatment and prognosis

Outcomes with standard R-CHOP therapy are significantly poorer for DHL, with one series from British Colombia reporting 5-year PFS and OS of 18% and 27%, respectively. Accepting the caveats of retrospective data and possible under-utilisation of FISH, there is general acceptance that R-CHOP is insufficient therapy for these patients and there has been much focus on use of intensified immunochemotherapy regimens for DHL.

Evidence to support the use of intensified regimens for DHL includes a systematic review and meta-analysis of 11 retrospective studies whereby treatment with DA-EPOCH-R resulted in improved PFS in 394 patients with DHL compared to R-CHOP therapy. However, this PFS improvement did not translate to superior OS. A multicentre study of 159 patients with DHL reported that more intensive front-line therapy (DA-EPOCH-R, R-HyperCVAD, R-CODOX-M/IVAC) was associated with improved 3-year PFS compared with R-CHOP, but again no difference in OS was demonstrated.

DA-EPOCH-R has been prospectively evaluated in a single arm, phase II trial of 53 patients with untreated *MYC* rearranged aggressive B-cell lymphomas. With median follow-up of 55.6 months, 4-year event-free and overall survival were 71.0 and 76.% respectively, with similar outcomes between those with single *MYC* translocation and those with DHL/THL. The only randomised phase III study comparing DA-EPOCH-R to R-CHOP thus far had very low numbers of patients with DHL/THL so no definitive conclusions could be drawn from this data on potential superiority of DA-EPOCH-R.

The UK NCRI phase 2 study of R-CODOX-M/R-IVAC in high-risk DLBCL patients included 59/116 patients where FISH results were available – 12% of these cases had DHL. The outcomes for the DHL patients were similar to the whole study cohort which had 2-year PFS/OS of 67.9 and 76.0%, respectively. This regimen has significant toxicity (TRM 4.3% in this study) but is used in some centres for DHL, particularly as it incorporates CNS penetrating agents.

Given the poor outcomes in DHL treated with R-CHOP, many centres previously advocated consolidative therapy with high-dose chemotherapy and ASCT in first complete response (CR). However, a large US analysis of patients with DHL who achieved first CR following completion of front-line therapy (with either R-CHOP or more intensive regimens) demonstrated that ASCT did not improve relapse-free survival (RFS) or OS. Patients treated with R-CHOP had inferior RFS compared to those receiving intensive therapy but similar OS, and sub analyses suggested a benefit of ASCT specifically for the R-CHOP treated group. However, these data do not advocate an approach of R-CHOP induction followed by ASCT as patients are less likely to achieve CR with R-CHOP, but importantly there does not appear to be benefit of ASCT consolidation in those achieving CR and in those undergoing intensive induction.

Conclusion and future directions

Although intensified front-line therapy has not been definitively proven in a prospective trial to improve outcomes for DHL/THL, the outcomes with R-CHOP alone are unsatisfactory and there is sufficient cumulative evidence to support intensified regimens for these patients. With more sophisticated molecular diagnostic techniques and classification, recognition of these high-risk patients is likely to move beyond simply using FISH to identify the three described translocations. Recent studies have used RNA sequencing to identify a gene expression signature associated with the presence of DHL. When applied to large cohorts of DLBCL patients, most cases of DHL (by FISH) are identified with this technique but many non-DHL cases exhibiting similar high-risk features and poor outcomes are also picked out. Identifying a more homogenous group of poor-risk patients in this way may enable focussed attempts at improving outcomes with prospective trials incorporating novel agents, intensified immunochemotherapy, or both.

Primary mediastinal (thymic) large B-cell lymphoma

Primary mediastinal large B-cell lymphoma (PMBL) is an aggressive lymphoma that accounts for 2–4% of all NHLs. PMBL is recognised as a separate entity in the WHO classification of

lymphoid neoplasms, with distinct clinic-pathological features from DLBCL. It characteristically presents with a large anterior mediastinal mass frequently accompanied by clinical complications such as pericardial or pleural effusions and superior vena cava obstruction as a result of local intrathoracic tumour invasion. Extra-thoracic involvement and bone marrow infiltration are rare, although at relapse, there is a significantly high incidence of extra-nodal site involvement. The median age at presentation is in the third to fourth decade with a female predominance. Interestingly, PMBL shares not only clinical and pathological features with classical Hodgkin lymphoma (cHL) but also molecular similarities, with GEP demonstrating greater resemblance with cHL than with DLBCL.

Genetic and molecular features

The cell-of- origin of PMBL is unclear although the pattern of somatic hypermutation of typical gene targets such as *BCL6* and the immunoglobulin heavy chain gene (IgVH) and GEP supports derivation from thymic B-cells with a germinal centre or post-germinal centre origin. Key molecular pathways and biological mechanisms involved in PMBL include deregulated NF-κB, Janus kinase–signal transducers and activators of transcription (JAK-STAT) signalling and immune surveillance. A number of these genetic and molecular aberrations are shared by cHL.

Gains and amplification of chromosome 2p16.1 containing the gene locus for *REL,* occur in over 50% of patients, with increased NF-κB activity seen in these cases. Additionally, truncating mutations and deletions of *TNFAIP3*, a negative regulator of NF-κB signalling, occurs in 36% of PMBL, promoting constitutive NF-κB activation.

The *JAK2* gene at chromosome 9p24.1, is amplified in up to 75% of PMBL cases and is a major molecular hallmark of the disease. Other components of the JAK-STAT signalling pathway are altered in PMBL including recurrent mutations in *PTPN1*, encoding a protein tyrosine phosphatase, the tumour suppressor gene, *SOCS1* and *STAT6*.

MHC Class II antigens such as HLA-DR are downregulated on the surface of PMBL cells and correlate with a diminished number of infiltrating cytotoxic T-cells and an inferior outcome. Translocations in the transcriptional regulator of MHC II class expression, *CIITA*, occur in 38% of PMBL and shown to suppress HLA class II expression *in vitro*. Highly recurrent genomic rearrangements involving T-cell anergy-inducing programmed death ligands (PDL1 and PDL2) lend further support to the role of escape from immune surveillance as a feature of PMBL.

Treatment and prognosis

Initial therapy is critical in treating PMBL; however, there has been limited prospective trial activity in PMBL making it difficult to establish a standard of care. This is further compounded by the dilemma of how to deliver a therapy with the highest cure rate yet minimise long-term morbidity and sequelae in this young population. Currently, therapies for PMBL consist of a multi-agent anthracycline- and rituximab-containing backbone. Multiple retrospective studies have suggested improved response rates and remission duration with the addition of rituximab, as well as prospective data from PMBL patients treated within the MInT trial. There remains a lack of consensus worldwide on the optimal chemotherapy backbone for PMBL. R-CHOP and DA-EPOCH-R are the two most widely used worldwide, although there has been no randomised controlled trial comparing the two regimens specifically in PMBL.

Data supporting R-CHOP in PMBL is derived from either retrospective analyses or from post-hoc subgroup analyses from large DLBCL trials. The MiNT trial of CHOP-like therapy with or without rituximab (Table 27.3) included 87 patients with PMBL. The rituximab arm had significantly improved EFS (78 versus 52%) and a trend towards improved OS (89 versus 78%) compared with CHOP only. The majority (61%) received involved field radiotherapy. The UK NCRI R-CHOP 14 versus R-CHOP 21 trial included 50 patients with PMBL, with 5-year PFS and OS of 79.8 and 83.8%, respectively. There was a trend towards survival benefit in the R-CHOP 14 arm in this study, and recent non-randomised data from the IELSG37 study supports the recommendation of R-CHOP on a 14-day rather than 21-day cycle in PMBL.

DA-EPOCH-R has been evaluated prospectively in PMBL, albeit only in a single centre phase II trial of 51 patients. The outcomes in this study were very encouraging with 5-year EFS of 93% and OS 97%, although toxicity was significant with 13% of cycles resulting in admission for neutropenic fever. Only two patients received radiotherapy due to locally progressive disease, suggesting that use of DA-EPOCH-R may obviate the need for consolidative radiotherapy if CMR is attained. More recently, the IELSG37 randomised question has shown that radiotherapy does not provide a significant benefit to patients achieving a CMR after R-CHOP. The vast majority of patients with a positive PET scan did receive radiotherapy however and omission of radiothearpy in, for example, patients with a Deauville 4 response, remains controversial.

Relapse of PMBL is infrequent compared to DLBCL with the majority of treatment failures occurring within the first 6–12 months after therapy completion, and rarely beyond 2 years. A repeat biopsy is advocated to confirm histology at the time of recurrence. Broadly speaking, the approach for PMBL relapse, similar to DLBCL, is salvage therapy followed by consolidation with ASCT in those deemed fit enough, although radiotherapy should be considered if omitted from initial therapy where relapse is localised. Patients with PMBL were included in the initial trials of the CAR T-cell products axicabtagene ciloleucel and lisocabtagene maraleucel and therefore these agents can be considered where available, with indications in line with DLBCL. In keeping with the frequent expression of PD-L1 in PMBL, there is also some promising data with the use of immune checkpoint inhibitors (e.g. pembrolizumab, nivolumab) as well as Brentuximab in the relapsed/refractory setting.

Primary central nervous system lymphoma

Primary central nervous system lymphoma (PCNSL) is rare form of NHL comprising 2–4% of all central nervous system (CNS) tumours. It encompasses lymphoma exclusively involving the brain, spinal cord, eyes, meninges and cranial nerves, with 90–95% classified histologically as DLBCL. Depending on the location involved, PCNSL can present with a variety of symptoms. Intracranial lesions are the most common manifestation of PCNSL and the majority present with focal neurologic symptoms, but nonspecific neuropsychiatric symptoms are also observed. Primary vitreoretinal lymphoma can present with blurred vision and symptoms mimicking uveitis.

The median age of presentation of PCNSL is around 70 years. The majority of PCNSL are sporadic and an increasing incidence has been recognised in recent years, particularly in older patients (>60 years). A minority are attributable to immunosuppressed states, including HIV infection or iatrogenic immunosuppression following organ transplantation, and the tumours in this setting are biologically different and usually EBV positive. In the era of effective ART, the frequency of HIV-associated PCNSL has diminished.

The involvement of critical sites within the CNS presents both diagnostic and therapeutic challenges, with outcomes inferior to systemic DLBCL. Neurocognitive dysfunction and impaired performance status are frequent at clinical presentation, whilst histological confirmation is inherently risky and often yields small tissue biopsies. Moreover, choice of cytotoxic therapy is limited by the inability of many drugs employed for systemic NHL treatment to penetrate the blood–brain barrier (BBB) efficiently. Since the initial description of PCNSL in 1975, treatment algorithms have evolved from whole-brain radiotherapy (WBRT) as a single-modality treatment towards a multiagent, high-dose, MTX-based chemotherapy approach and consideration for consolidation with chemotherapy with or without ACST in fit patients. WBRT is used less commonly in frontline consolidation and reserved more often to treat relapsed disease. Given the rarity of PCNSL, data from randomised studies are scarce, but a number of clinical trials performed over the last decade have addressed key questions addressing optimal induction, consolidation and maintenance strategies.

Diagnosis and prognosis

The diagnosis of CNS lymphoma can be a particular challenge because of lesional response to corticosteroids and radiological features that are shared with other pathologies. Multimodal gadolinium-enhanced MRI remains the standard of care for diagnosis and therapeutic monitoring of PCNSL. At initial diagnosis, PCNSL in immunocompetent individuals classically demonstrates avidly enhancing lesions (up to 60%, 75% of which are multiple). Most are supratentorial (80%), in a periventricular or deep cerebral white matter distribution. The majority of PCNSL are diagnosed via stereotactic biopsy or, less commonly, by flow cytometric analysis of CSF. A number of biomarkers, such as interleukin-10 (IL-10) and myeloid differentiation primary response L265P mutation (mut-*MYD88*), detected in CSF, show promise in detecting PCNSL both at initial diagnosis and relapse. Recently, the value of detection of ctDNA at diagnosis and response assessment has been highlighted. Whole body 18FDG PET-CT has an increased sensitivity for detection of systemic DLBCL over conventional CT staging and has an important role in the exclusion of systemic lymphoma at presentation.

Age and baseline performance score are the main determinants of prognosis in PCNSL and both are incorporated into prognostic scores.

Genetic and molecular features

The pathobiology of PCNSL has arisen through observations from small and heterogeneously treated populations. The majority of PCNSLs type as the ABC cell-of- origin. There is a high incidence of recurrent mutations in genes that drive BCR and NF-κB signalling including *MYD88* and *CD79B*. The serine/threonine kinase, PIM1, involved in cytokine signalling and cell proliferation is also frequently mutated as are deletions of 6p21, involving the HLA locus (56–79%), representing a potential mechanism for immune escape. The genetic features of PCNSL align very closely to the newly identified MCD/C5 molecular group in DLBCL. Notably, EBV-associated PCNSL usually occurring in the context of immunosuppression are rare but have a distinct biology with nearly complete absence of the above genetic features described.

Treatment – remission induction

Chemotherapy regimens incorporating HD-MTX are considered the standard of care as induction therapy for newly diagnosed PCNSL, achieving high rates of initial response and improved survival outcomes when combined with other cytostatic agents. There is general consensus that MTX should be administered as a rapid infusion (2–4 hours) at a dose of at least 3 g/m^2 to maximise therapeutic CSF concentrations, at an interval of 14–21 days. Over recent years, a growing number of clinical studies investigating intensive immunochemotherapy approaches have included older patients. For example, the MATRix regimen comprising rituximab, HD-MTX, cytarabine and thiotepa has proven to be effective in patients up to the age of 70 years in the IELSG32 trial. Other popular regimens include HD-MTX, carmustine, etoposide and prednisone (MBVP); HD-MTX, vincristine, and procarbazine (MVP); and HD-MTX, temozolomide, and rituximab (R-MT).

Several studies have evaluated the additional value of BBB-penetrating alkylating agents, such as temozolomide and

procarbazine, providing non-cross-resistant agents that, unlike antimetabolite chemotherapy, are also cytotoxic to cells in G0 of the cell cycle.

Treatment: consolidation

One of the paradigms in the treatment of patients with PCNSL is that induction therapy should be followed by consolidation treatment to achieve long-term remissions. The rationale for HDT-ACST in PCNSL is the delivery of BBB-penetrating agents into the CNS, in several-fold higher concentrations compared to conventional therapy, which cannot be achieved without autologous stem cell rescue. Various single arm and randomised trials provide a robust body of evidence for the use of HDT-ASCT in younger PCNSL patients <65-70 years with 3–5 years survival ranging between 69 and 87%. A recent retrospective study which included 603 patients demonstrated superiority of thiotepa-based conditioning associated with better 3-year PFS (75 versus 58% with BEAM), notably the upper limit of age for HDT-ASCT was 77 years.

WBRT following HD-MTX-based induction chemotherapy is associated with delayed neurotoxicity, particularly with increasing age and increased doses of WBRT, and this is associated with significant morbidity and mortality. There is increasing reluctance to use this consolidation therapy at first response although the efficacy and toxicity of reduced dose (rd) WBRT (23.4 Gy) with no boost in patients with radiographic CR after induction has been assessed in some prospective and retrospective studies. However, the safety of rdWBRT in elderly patients remains to be determined, since few patients older than 60 years completed neuropsychological evaluation.

Consolidation with a nonmyeloablative chemotherapy has also been suggested as potentially less toxic, less resource-consuming consolidation strategy in patients with PCNSL. Two randomised Phase II trials have assessed the efficacy and toxicity of this strategy compared to HDT-ASCT and preliminary results from one study suggest less intensive chemotherapy consolidation may be less effective although more data is awaited.

However, with a median age of 68 years, a significant proportion of patients with PCNSL are not eligible for consolidation treatment. For this population, maintenance treatments have been and are being evaluated in several studies. Agents such as procarbazine, temozolamide, rituximab, obinutuzumab, nivolumab, ibrutinib and lenalidomide are being explored.

Relapsed PCNSL

While PCNSL is chemo and radiosensitive, relapse is as common as 50% in the first 2 years highlighting the need for improved durable therapies. Currently, there exists no standard treatment approach for patients with relapsed and refractory PCNSL. Published data are largely restricted to small, uncontrolled, retrospective studies, in which outcomes are influenced by a number of parameters, including prior therapies and response durations. In studies where eligible patients have proceeded to HDT-ASCT, available data support the use of thiotepa-based HDT-ASCT in relapsed PCNSL.

Studies addressing the efficacy of agents such as temozolomide, pemetrexed, bendamustine, topotecan, lenalidomide, pomalidomide, temsirolimus, ibrutinib, PI3K inhibitors, a dual PI3K/mTOR inhibitor and other agents targeting B-cell signalling are ongoing. Immunotherapies with CAR T-cells are also being evaluated in PCNSL.

Trials involving new therapies should include robust assessment of neurocognitive functioning and quality of life metrics as the minimisation of neurotoxicity is a primary goal in PCNSL treatment.

Burkitt lymphoma

Burkitt lymphoma (BL) was first described in Ugandan children by Denis Burkitt in 1958. BL is a highly aggressive mature B-cell lymphoma that constitutes less than 2% of all NHL diagnoses in the UK. It represents the most frequent B-cell lymphoma in children accounting for 30–50% of all paediatric lymphomas. There are three clinical variants recognised: endemic, sporadic and immunodeficiency-associated BL.

The endemic BL variant occurs predominantly in equatorial Africa and Papua New Guinea, mainly affecting children with an estimated incidence of 3–6 cases per 100,000 children per year and a peak incidence of 4–7 years. The risk factors for endemic BL are incompletely understood. However, EBV is widely accepted to be a contributing factor with the EBV genome detected in about 95% of endemic BL cases. Infection with plasmodium falciparum malaria has also been linked as a risk factor for endemic BL. The ubiquity of EBV and malaria in these regions suggest that these factors alone cannot explain all endemic BL cases. Sporadic BL is distributed worldwide, occurring mainly in children and young adults. EBV can be detected in about 30% of sporadic BL cases.

Presentation varies according to the clinical subtype but extranodal site involvement is a common feature. CNS involvement (typically leptomeningeal rather than parenchymal) may occur at presentation, particularly when there is advanced-stage disease. In endemic BL, the jaw and facial bones are characteristically affected. For sporadic BL, presentation with intra-abdominal masses is more common, with jaw tumours rarely seen. Lymph node presentation is seen more commonly in adults than children.

Genetic and molecular features

BL is derived from a germinal-centre cell-of- origin yet has a unique gene expression profile that distinguishes it from DLBCL. Recent characterisation has shown that BL most closely resembles

cells from germinal-centre dark zones, whereas the GCB subtype of DLBCL is more akin to cells from germinal-centre light zones.

The molecular hallmark of BL is the translocation t(8;14)(q24;q32) or its variants t(2;8) and t(8;22), occurring in nearly all affected individuals irrespective of the clinical variant. These chromosomal translocations juxtapose the *MYC* oncogene at 8q24 with one of the three immunoglobulin loci partners: Ig heavy chain (IGH) at 14q32, kappa light chain (IGK) at 2p12 or lambda light chain (IGL) at 22q11, leading to deregulation of MYC expression. While *MYC* rearrangement is a diagnostic feature of BL, it is not specific and occurs less commonly in DLBCL and other aggressive lymphomas. A recently discovered entity that shares morphological, phenotypic and gene expression profiles to sporadic BL but lacks MYC rearrangement was included in the WHO 2016 update, Burkitt-like lymphoma with 11q aberration. These lymphomas are characterised an 11q aberration (typically proximal gains and telomeric losses, see Figure 27.2), complex karyotypes and a mutational landscape that differs from sporadic BL. The WHO 2022 update updated the terminology subtly to reflect this, classifying such cases as 'High-grade B-cell lymphoma with 11q aberrations'.

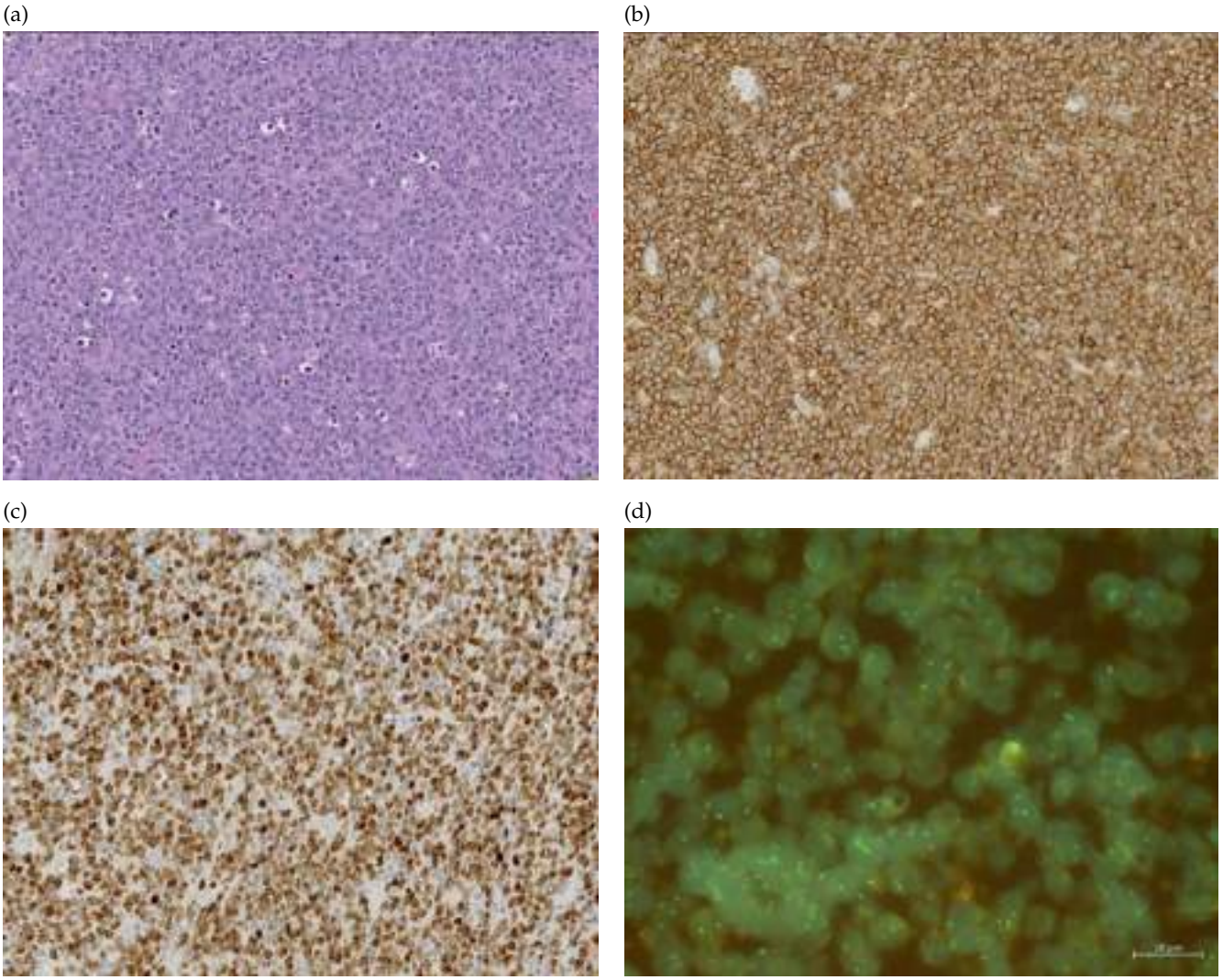

Figure 27.2 High-grade B-cell lymphoma with 11q aberration (previously known as Burkitt-like lymphoma with 11q aberration in WHO 2016 classification). (a) H&E staining demonstrating high-grade lymphoma morphology with 'starry-sky' appearance due to presence of scattered tangible-body laden macrophages. (b) Uniform positivity of CD20 on immunohistochemistry confirming B-cell lineage. (c) Ki-67 immunohistochemistry positive in >90% of cells. (d) FISH using 11q gain/loss triple-colour probes showing two to three green signals in the 11q23.3 region but only one orange signal at the 11q24.3 region, confirming the presence of typical 11q gain-loss pattern. (Source: Au-Yeung RKH, Padilla LA, Zimmermann M *et al.* (2020) Experience with provisional WHO-entities large B-cell lymphoma with *IRF4*-rearrangement and Burkitt-like lymphoma with 11q aberration in paediatric patients of the NHL-BFM group./JOHN WILEY & SONS, INC. / CC BY-NC 4.0.)

Current models for BL assume that IG-MYC translocation is an early genetic event and additional cooperating oncogenic events are required to drive lymphomagenesis. *MYC* and *TP53* mutations occur in approximately 60% and 40% of BL cases, respectively. Recent genomic sequencing studies have revealed novel activating mutations in the transcription factor, *TCF3* and inactivating mutations in its negative regulator, *ID3*, affecting up to 70% of cases. Additionally, activating mutations in *CCND3* (encoding cyclin D3) have been identified in 38% of sporadic BL. Together, these recurrent oncogenic mutations cooperate with *MYC* to promote proliferation, survival and growth of BL cells.

Treatment and prognosis

Until recently, estimating prognosis for adults with BL was challenging because the international prognostic index (IPI) used for other aggressive lymphomas lacked prognostic discrimination in BL. However, a recent multicentre analysis of >1000 patients with BL identified variables showing the strongest prognostic association with PFS, resulting in creation of the BL International Prognostic Index (BL-IPI). The BL-IPI is made up of the following four risk factors; age ≥40 years, performance status ≥2, serum LDH >3× upper limit of normal and CNS involvement. The score was validated in an external data set whereby 3-year PFS estimates were 96, 82 and 63% for low (score 0), intermediate (score 1) and high risk (score ≥2) groups, respectively. This scoring system will be useful in the clinical setting for prognostication and also for designing clinical trials in BL, which will largely focus on reducing toxicity for low-risk patients who have excellent long-term outcomes and incorporating novel agents for poor-risk groups.

Therapies in BL are typically more intensive than DLBCL. Due to the high proliferation rates of the tumour, a dose-intense, short-duration treatment strategy is employed to avoid rapid growth and development of resistance between chemotherapy cycles. Effective treatment of patients with BL is therefore based on a multi-drug combination chemotherapy of anti-metabolites and alkylating agents (e.g CODOX–M/IVAC regimen, hyper CVAD; Table 27.4) and includes drugs which highly penetrate the blood–brain barrier, such as methotrexate and cytarabine. Prospective trials in adults and children have now demonstrated that addition of rituximab to chemotherapy is associated with improved survival and this is now universally recommended in BL. With the above rituximab-containing regimens, CR and 3 year-PFS rates are in the region of 80–94% and 70–90% respectively. Addition of granulocyte-colony stimulating factors (G-CSF) is required in these regimens to maintain dose intensity and reduce the duration of severe neutropenia.

In recent years, the less dose-intense regimen DA-EPOCH-R has been used in BL with good clinical outcomes. A single arm, multicentre study published in 2020 investigated using a risk-adapted approach with DA-EPOCH-R in 113 adults with untreated BL. Low-risk patients received three cycles with no CNS prophylaxis, and high-risk patients received six cycles with intrathecal CNS prophylaxis (or extended intrathecal treatment if leptomeninges involved). At median follow-up of 59 months, EFS/OS were 84.5 and 87.0% respectively, and EFS was 100 and 82.1% in low- and high-risk patients. Notably, involvement of the CSF was the most significant risk factor for early TRM or treatment failure in this study, and a recent multicentre retrospective analysis of 641 patients demonstrated that intensive regimens containing CNS penetrating agents were associated with lower rates of CNS relapse compared to DA-EPOCH-R. Larger studies are required to evaluate this regimen in adult BL, but it may be an acceptable option for patients who may not tolerate more intensive regimens.

The risk for tumour lysis syndrome (TLS), especially after initiating chemotherapy, is relatively high, reflecting the high proliferation rate of tumour cells. Vigorous hydration with alkalinisation, accompanied by administration of recombinant urate oxidase (rasburicase) (or a uric acid synthesis inhibitor such as allopurinol) is very important in preventing/reducing the severity of this complication (see also Chapter 55). Hypercalcaemia is also frequent at presentation and needs appropriate therapy with hydration and bisphosphonates.

Relapses in BL typically occur within the first 12 months following treatment. Although BL is a highly FDG-avid tumour, there is currently no consensus on its usefulness for response assessment, and this requires further exploration. There remains a lack of improvement in the outcome of patients older than 60 years, who make up a quarter of BL diagnoses and experience a higher rate of adverse events and for which the standard dose-intense regimens may be unsuitable and alternative approaches are warranted. Newer molecularly-targeted therapies may be beneficial in this cohort of patients, as well as in patients with relapsed/refractory disease who historically have an extremely poor prognosis.

Burkitt lymphoma in HIV-positive patients

A number of studies have demonstrated the feasibility of administering intensive chemotherapy regimens, such as CODOX-M/IVAC, with or without Rituximab, to HIV-positive patients with BL. These studies report a 2-year EFS of approximately 60%, similar to that observed in HIV-negative patients treated with the same regimen, with no increase in toxicity and prompt immune recovery suggesting that a uniform approach to treatment of BL should be used, regardless of HIV status. The DA-EPOCH-R regimen has also been shown to be effective in this cohort, but the lack of CNS penetrating agents included is a concern with this therapy. A recent large retrospective analysis of 249 patients with HIV-related BL found that the incidence of CNS relapse was higher with DA-EPOCH compared to other regimens, and the most effective regimen with regards to PFS

appeared to be CODOX-M/IVAC. The same study demonstrated for the first time that prognostic factors for patient outcome were associated with lymphoma characteristics, rather than HIV-related features.

Post-transplant lymphoproliferative disorder

Post-transplant lymphoproliferative disorder (PTLD) represents a spectrum of disorders resulting from lymphoid proliferations occurring as a result of immunosuppression following allogeneic solid organ or haematopoietic stem cell transplantation. The WHO 2016 classification categorises PTLD into non-destructive PTLDs (plasmacytic hyperplasia, infectious mononucleosis, florid follicular hyperplasia), polymorphic PTLD, classical Hodgkin lymphoma PTLD and monomorphic PTLDs. Monomorphic PTLDs cannot be distinguished from lymphomas with a similar lineage and cell-of- origin in immunocompetent patients, therefore subclassification of these types should be the same (i.e. DLBCL, Burkitt lymphoma, plasma cell myeloma, plasmacytoma, T-cell neoplasms). EBV causes the majority of these disorders: impaired T-cell function leads to proliferation of latently EBV-infected polyclonal B-cells, whereas subsequent genomic mutations result in monoclonal/monomorphic PTLD. Of note, EBV-negative PTLD can occur and has been documented in up to 30% of PTLD in some series.

The optimal treatment of PTLD is still not clearly defined with most published data in the form of case series. Therapeutic options in PTLD include immunosuppression reduction, chemotherapy and immunotherapy. Management should be undertaken in conjunction with a transplant physician, particularly with respect to immunosuppression reduction and surveillance of graft function. Patients may respond to reduction in immunosuppression, especially those who develop PTLD within 1 year from transplant (ORR 80 versus 10% in patients who developed PTLD beyond 1 year). Case series and Phase II studies of rituximab monotherapy following failure of reduction of immunosuppression have confirmed efficacy in inducing remission in a proportion of patients (44–65%) with PTLD. Toxicity appears to be low, but significant numbers of patients progressed on therapy or relapsed after rituximab, some died of lymphoma and many required intensification to chemotherapy.

The prospective phase 2 PTLD-1 trial previously demonstrated the efficacy and safety of sequential therapy (rituximab followed by CHOP chemotherapy) for solid organ transplant recipients with CD20-positive PTLD unresponsive to reduction of immunosuppression. Despite supportive treatment including mandatory G-CSF support and antibiotic prophylaxis, infective toxicity was still significant with a TRM of 13%. The subsequent PTLD-1/3 trial introduced a response-adapted schema whereby patients were assessed following 4 weekly rituximab doses with interim CT. Those obtaining a CR were considered 'low risk' and received four further doses of weekly rituximab only, while those not obtaining CR were deemed 'high risk' and switched to four cycles of R-CHOP-21. The median OS was identical to the PTLD-1 trial but TRM was reduced to 8%. The treatment algorithm used in PTLD-1/3 is now widely employed; however, patients with clinically aggressive lymphoma and critical organ compromise are often treated with R-CHOP-21 upfront. Prospective trials are ongoing investigating incorporation of novel agents, e.g. ibrutinib, to front-line therapy.

Patients with relapsed/refractory PTLD post-R-CHOP therapy have poor long-term survival. There is data to support use of intensive second-line chemotherapy and ASCT in this setting, but patients must be selected very carefully for this approach given the underlying comorbidity inherent in this group. Anti-EBV cytotoxic T lymphocytes have been used for chemorefractory disease but their availability is restricted, and their infusion is associated with regulatory challenges.

Other rare aggressive B-cell lymphoma subtypes

Intravascular large B-cell lymphoma

Intravascular large B-cell lymphoma is an extremely rare form of NHL and is characterised by an accumulation of large neoplastic B-cells within the lumen of blood vessels. These lymphomas preferentially affect the elderly and often involve the CNS (approximately 30% of patients), lungs and skin. Prognosis is generally poor. Patients presenting with only skin lesions appear to have a significantly better survival than patients with other clinical presentations (3-year OS: 56 versus 22%). Multi-agent chemotherapy using agents with higher CNS bioavailability is the preferred mode of treatment.

T-cell/histiocyte-rich large B-cell lymphoma

T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL) may arise de novo, but more commonly occurs as a high-grade transformation of underlying nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). As the name suggests, THRLBCL is characterised by a limited number of scattered, large B-cells embedded in a background of abundant T cells and histiocytes. Pathologically, it can be difficult to distinguish from NLPHL, and therefore clinical correlation is key to diagnosing the high-grade transformation. It has a strong male predominance and typically occurs in middle-age, often presenting with advanced-stage disease and extranodal involvement. It should be treated identically to DLBCL-NOS, but often behaves in an aggressive manner with poor outcomes.

Primary cutaneous diffuse large B-cell lymphoma, leg type

This entity comprises 20% of all primary cutaneous B-cell lymphomas and typically occurs in older women. It classically presents with red or bluish-red rapidly growing tumours on one or both lower legs, but 10–15% of cases arise at other sites and dissemination to extracutaneous sites is common. The neoplastic B-cells have a gene expression profile of ABC subtype *DLBCL*, and *MYD88* L265P mutations are found in up to 60% of cases. Historically, outcomes have been reported as being very poor, although this may in part reflect the preponderance for older patients. Treatment is often similar to approaches for limited-stage DLBCL, involving R-CHOP for those deemed fit enough followed by involved field radiotherapy.

EBV-positive diffuse large B-cell lymphoma

This disease was formerly called EBV-positive DLBCL of the elderly as it typically occurs in patients over the age of 50, but the restriction to elderly patients has been removed from the WHO 2016 and 2022 classifications. It is a distinct entity which should be distinguished from other well-defined lymphomas that may be EBV-positive (e.g. plasmablastic lymphoma, DLBCL associated with chronic inflammation, PTLD). The increased incidence in older patients is thought to relate to immunosenescence. Advanced-stage disease and extranodal involvement are relatively common. Treatment approach is identical to that of DLBCL, NOS and prognosis varies according to age with older patients tending to have poorer outcomes.

Diffuse large B-cell lymphoma associated with chronic inflammation

This is a neoplasm occurring in the setting of longstanding chronic inflammation and is uniformly associated with EBV. It typically arises in patients with a lengthy history of pyothorax, but other reported sites of involvement include bone (especially femur), joints, and periarticular soft tissue. DLBCL associated with chronic inflammation is an aggressive disease and confers a poor prognosis. Immunochemotherapy and radiotherapy are typically employed, although there have been reports of complete tumour resection resulting in good long-term outcome.

Plasmablastic lymphoma

Plasmablastic lymphoma (PBL) is a rare, aggressive lymphoma subtype seen most commonly in immunosuppressed states, in particular with HIV infection, with cases in immunocompetent patients occurring rarely. PBL is thought to arise from post-germinal centre B-cells that are in transition towards plasma cell differentiation, as evidenced by loss of typical B-cell markers but strong expression of plasmacytic markers (e.g. CD138, MUM1 and BLIMP1). Approximately 60% of cases are associated with EBV infection, and 50% harbour *MYC* translocations.

PBL commonly arises in the oral cavity although may present with widespread, advanced-stage disease. Overall, outcomes are very poor, although early-stage disease and complete response to chemotherapy are associated with superior outcomes. There is no consensus regarding optimal treatment and many use CHOP but regimens used for other aggressive lymphomas are also employed, including CODOX-M/IVAC and DA-EPOCH. Given the plasmacytic differentiation of the tumour cells, the proteasome inhibitor bortezomib (commonly used to treat plasma cell disorders) has been added to regimens, with some small single-arm studies showing promising results.

Primary effusion lymphoma

Primary effusion lymphoma (PEL) is a rare disorder occurring almost exclusively in PLWH, characterised by a proliferation of monoclonal B-cells expressing the plasmacytic marker CD38 and often CD30. EBV co-infection is commonly found, but its role in the pathogenesis of PEL is not clear. As the name suggests, it typically presents with malignant effusions e.g. pleural, peritoneal or pericardial. Despite often presenting as limited-stage disease, PEL causes local destruction and has a dismal prognosis. There is a paucity of data to guide treatment, but intensive chemotherapy, such as DA-EPOCH is often attempted for patients deemed fit enough, with radiotherapy reserved for poorly responding patients or to provide symptomatic control.

HHV8-positive DLBCL

HHV8-positive DLBCL, NOS, most commonly arises in association with HHV8-positive multicentric Castleman disease (MCD) in the setting of immunosuppression. The disease usually manifests with rapidly enlarging lymph nodes and splenomegaly, often in patients with clinical features of MCD, and is associated with an extremely aggressive clinical course and refractoriness to chemotherapy.

Future directions

Over the last decade, we have seen huge advances in the field of aggressive B-cell NHL, from the application of sophisticated genetic and molecular analysis to improve subclassification of DLBCL, through to the application of novel cellular therapies for patients with relapsed/refractory disease. Although there continues to be no therapy proven to improve overall survival over R-CHOP for DLBCL, this is surely imminent as clinical trial design becomes more sophisticated and incorporates novel agents targeted to specific molecular subtypes. Response assessment using PET-CT in combination with circulating tumour DNA monitoring also has the potential to earlier identify patients

destined to do poorly with traditional immunochemotherapy. CAR T-cell therapy and bispecific antibodies are a hugely exciting advance and provide therapeutic options for patients who previously had no prospect of cure. It is likely that these therapies will move earlier in the treatment algorithm with time, although there is still much work to be done to improve response rates and reduce the substantial associated toxicity and cost of these treatments.

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CHAPTER 28

Multiple myeloma

28

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Epidemiology and aetiology

The annual age standardised incidence of MM is around 10 per 100,000. It represents approximately 1% of all malignant diseases and 15% of all haematological malignancies. The incidence of MM is lower in Asian populations and higher in blacks, almost twice that in whites; MM is slightly more frequent in men than in women. The median age at diagnosis is 65–70 years. Only 15 and 2% of the patients are younger than 50 and 40 years, respectively. The 5-year prevalence estimates according to the International Agency for Research on Cancer (IARC) are approximately 100,000 in North America and 138,000 in Europe.

The cause of MM is unknown although a number of risk factors have been explored. Radiation may play a role in some cases. An increased risk has been reported in farmers, particularly those who use herbicides and insecticides, and in people exposed to benzene and other organic solvents. However, the number of cases is small and more epidemiological data are needed to establish a significant relationship. MM and monoclonal gammopathy of undetermined significance (MGUS) a precursor state have been reported in familial clusters. A higher incidence is associated with older age, obesity, African or African–American ethnicity. Several studies have shown that patients with a first-degree relative with MM or the precancerous condition monoclonal gammopathy of undetermined significance are more likely to develop MGUS or MM than those without a family history. To date, many family linkage studies have been performed, providing strong evidence for an inherited component to the development of MM. The basis of this genetic contribution remains to be fully established, although studies have reported possible associations between certain genetic variations and myeloma risk. There is now clear evidence that most, if not all, myeloma cases are preceded by a previous MGUS.

Pathogenesis

MM is a B-cell malignancy characterised by the accumulation of terminally differentiated clonal plasma cells in the bone marrow, the production of a monoclonal immunoglobulin detectable in serum and/or urine and the presence of lytic bone lesions. In order to understand the pathogenesis of MM, it is important to review not only the molecular changes within the plasma cells involved in the development and behaviour of the malignant clone but also the mechanisms responsible for the interaction between the malignant plasma cells and their resident microenvironment, since they play a relevant role in bone destruction, tumour cell growth, survival, migration and drug resistance.

Cellular origin of myeloma cells

Normal differentiation from early B cells to plasma cells is characterised by three B-cell-specific DNA remodelling mechanisms that modify immunoglobulin genes: VDJ rearrangement, somatic mutation and class switch recombination. Rearrangements of the *immunoglobulin* genes of B-cell precursors to form a B-cell receptor (*BCR*) occur in the bone marrow, while antigen recognition, selection, somatic hypermutation and class switch recombination take place in germinal centres

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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within the lymph node, spleen or Peyer's patches. Sequence analysis of the immunoglobulin VH gene support the post-germinal centre origin of myeloma cells, which have successfully completed somatic hypermutation (without intraclonal variation) and IgH switching, before migrating to the bone marrow, where they will interact with stromal cells before finally differentiating into long-lived plasma cells.

Genomic abnormalities

Genome instability is a prominent feature of myeloma cells and in fact, almost all cases of MM are cytogenetically abnormal. Genomic abnormalities can be categorised as chromosomal translocations, mainly involving the IGH locus on chromosome 14q32, copy number abnormalities, mutations and epigenetic modifications.

IGH translocations

A primary event in many kinds of B-cell malignancy is dysregulation of an oncogene that, as a result of translocation to the *IGH* locus (14q32) or, somewhat less often, the *IGL* locus (κ 2p11 or λ 22q11), is juxtaposed near one of the potent immunoglobulin enhancers. In MM, *IGH* translocation may be classified into primary or secondary. Primary *IGH* translocations occur as initiating events during the pathogenesis of MM, whereas secondary translocations are involved in progression. Most primary *IGH* translocations result from errors in B-cell-specific DNA modification processes, mostly *IGH* switch recombination or, less often, somatic hypermutation. The breakpoints occur mainly within or immediately adjacent to *IGH* switch regions or JH regions. In contrast, secondary translocations are mediated by other kinds of recombination mechanism that do not specifically target B-cell-specific DNA modification processes. Unlike other B-cell malignancies, in MM there is a marked diversity of chromosomal loci involved in *IGH* translocations. About 40% of MM tumours have *IGH* translocations involving five recurrent chromosomal patterns (Figure 28.1): 11q13 (*CCND1*), 4p16 (*FGFR/MMSET*), 16q23 (*MAF*), 6p21 (*CCND3*) and 20q11 (*MAFB*).

The prevalence of t(11;14) according to interphase fluorescence *in situ* hybridisation (FISH) analysis is 15–20% and is readily detectable by karyotyping. As a result of the translocation, *CCND1* is juxtaposed to the powerful *IGH* 3′ enhancer(s) on der(14), and its expression is dysregulated, as indicated by gene expression profiling and reverse transcriptase polymerase chain reaction (RT-PCR) in 100% of MM cases with t(11;14).

The t(4;14) translocation is identified in approximately 15% of MM cases using FISH analysis but cannot be detected by karyotyping techniques. This translocation results in the simultaneous deregulation of the fibroblast growth factor receptor 3 (*FGFR3*) gene on der(14) and the multiple myeloma SET domain (*MMSET*) gene on der(4). FGFR3 is one of the high-affinity tyrosine kinase receptors for the FGF family of ligands. Both *FGFR3* and *MMSET* genes are not normally expressed in plasma cells but are over-expressed as a result of t(4;14). However, gene expression profiling and RT-PCR analysis have shown that only 75% of MM cases with t(4;14) display simultaneous over-expression of MMSET and FGFR3. In the remaining 25% of cases, only MMSET is upregulated and the lack of FGFR3 expression is linked in most cases to loss of the *FGFR3* gene on der(14). These data suggest that *MMSET* may be the critical transforming event in MM harbouring t(4;14), whereas *FGFR3* could be dispensable. In some cases, (10%) the translocated *FGFR3* contains activating mutations that may be involved in MM progression.

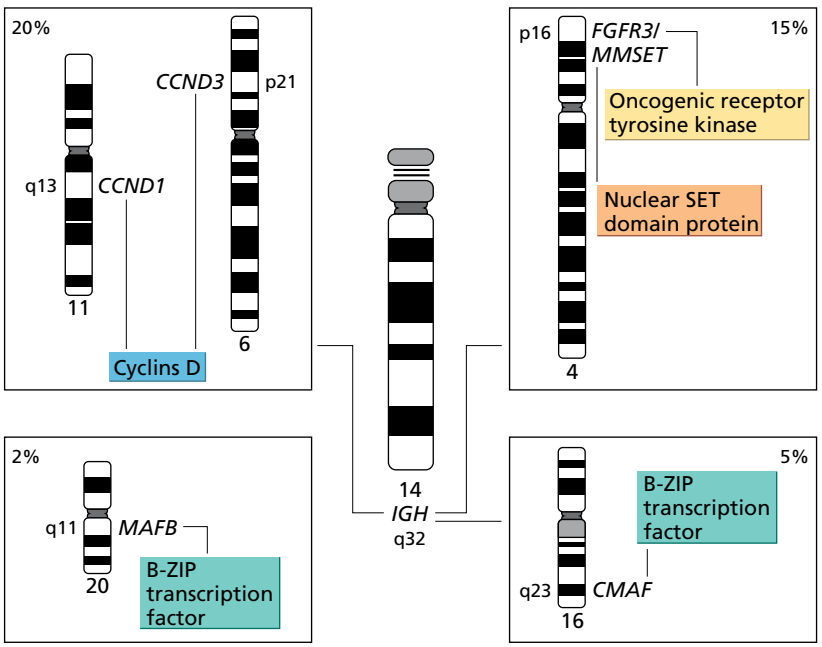

Figure 28.1 *IGH* translocations in multiple myeloma.

The incidence of t(14;16) is 5–10%. The breakpoints on 16q23 occur over a region 550–1350 kb centromeric to *MAF*. Taking into account such a long distance, it is still an open question whether or not *IGH* may act as enhancer for *MAF* in this translocation. Moreover, over-expression of MAF is observed in half of myeloma cases, while the prevalence of t(14;16) is low. The t(6;14) translocation has been found in a low proportion (3%) of MM cases. Using microarray analyses, high levels of cyclin D3 mRNA have been shown in cases with t(6;14) detected by FISH. The t(14;20) translocation leads to deregulation of *MAFB* (20q23), which, like *MAF*, encodes a B-ZIP transcription factor, but in contrast to t(14;16), *MAFB* translocations have structural features that indicate they are secondary translocations.

Gains and losses of chromosomal material

Almost all MM cases are aneuploid, as evidenced by the measurement of DNA content by flow cytometry and cytogenetic techniques. Patients with MM may be grouped into two major categories, according to ploidy status assessed by karyotyping: the hyperdiploid group (more than 46/47 chromosomes) and the non-hyperdiploid group, composed of hypodiploid (up to 44/45 chromosomes), pseudodiploid (44/45 to 46/47) and near tetraploid (more than 74) cases. Non-hyperdiploid MM is characterised by a very high prevalence of *IGH* translocations involving the five recurrent partners. Likewise, monosomy/deletion 13 and gains on 1q occur predominantly in non-hyperdiploid MM. In contrast, the hyperdiploid group is associated with recurrent trisomies involving odd chromosomes (3, 5, 7, 9, 11, 15 and 19) and with a low incidence of structural chromosomal abnormalities. Similar associations have been observed in analysing DNA content by flow cytometry.

The loss of chromosome 13 is the most common monosomy in MM (40–50% of newly diagnosed patients). This abnormality shows a strong association with t(4;14) and t(14;16), deletion of 17p and gains on 1q. Chromosome 17p deletion, which includes loss of *TP53*, occurs at a lower frequency in newly diagnosed MM (up to 10%), although the proportion is higher in advanced stages of the disease. Furthermore, 17p deletion is associated with extramedullary MM. Conventional cytogenetics, FISH and comparative genomic hybridisation analysis have all demonstrated that lesions of chromosome 1 are the most common abnormalities in MM; mostly they are 1q gains, as the result of tandem duplications and jumping segmental duplications of the chromosome 1q band. Large cooperative group studies have demonstrated that 1p losses (especially 1p22 and 1p32 deletions) are also frequent in MM patients.

Mutations detected by whole-genome sequencing

Whole-genome sequencing (WGS) strategies have shown that there are approximately 35 non-synonymous mutations per myeloma sample. However, only a few recurrently mutated genes have been detected, apart from the well-known mutations in the MAPK pathway. This is in agreement with other haematological malignancies, such as acute myeloid leukaemia, but is in contrast to hairy cell leukaemia and Waldenstrom's macroglobulinaemia, in which single unifying mutations are seen, *BRAF* and *MYD88*, respectively. Frequently mutated genes are *KRAS* and *NRAS* (20%) followed by *TP53*, *DIS3*, *FAM46C* and *BRAF*, which are all mutated in up to 10% of patients. All other mutations were observed in about 5% of patients with two or more mutations in genes involved in the MAPK pathway noted in some patients. The impact of all mutations apart from *TP53* mutation on survival and response to therapy remains unclear.

Epigenetic modifications

Compared to the considerable data available on genomic changes in myeloma patients, there is limited evidence about the epigenetic changes involved in MM pathogenesis. The most relevant epigenetic change revealed so far is aberrant methylation and up to 5% of genes relevant in DNA and histone methylation mutated in myeloma patients. Enhancer of Zeste homologue 2-*EZH2* encodes part of PRC2 complex catalytic component overexpression has been described within the context of MM, demonstrating its negative prognostic value: and increased EZH2 during disease progression, from MGUS, to myeloma has been reported. MMSET/NSD2 acts as a histone-modifying enzyme, and its abnormal expression in MM is driven by the t(4;14). *NSD2* interacts with both histone-H3 and -H4, thus leading to H3K36-dimethylation and H4K20-trimethylation; of note, it also favours the function of histone deacetylase-1, -2, and histone demethylase LSD1. These changes lead to myeloma progression. The histone deacetylase inhibitor (HDAC), Panobinostat, is approved for treatment of relapsed myeloma patients in combination with proteasome inhibitors.

Late genetic events

Some genetic changes in MM, such as secondary translocations, mutations, deletions and epigenetic abnormalities, are considered late oncogenic events and are associated with disease progression. Gain of 1q21 noted in up to 40% of patients has been observed to be amplified with additional copies of 1q at disease progression, in a proportion of patients. Dysregulation of *MYC* is a paradigm for secondary translocations in MM. Most karyotypic abnormalities involving *MYC* correspond to complex translocations and insertions that often are non-reciprocal and frequently involve three different chromosomes. Activating *RAS* mutations are considered molecular markers of disease progression. Thus, the prevalence of activating *KRAS* and *NRAS* mutations is over 75% in MM cases at relapse. *TP53* inactivation, via either deletion or mutation, seems to be more frequently associated with disease progression. Methylation is an epigenetic change that has been described in MM and acts as an inactivating mechanism of the tumour-suppressor genes *CDKN2B*

Table 28.1 Prognostic value of the main chromosomal abnormalities of multiple myeloma.

Abnormality	Prognostic value
t(4;14)	Worse prognosis
Del(17p)	Worse prognosis
Del(1p32)	Worse prognosis
1q gains	Worse prognosis
t(14;16)	Worse prognosis
Hyperdiploidy	Better prognosis
t(11;14)	Neutral prognostically

and *CDKN2A*. Although it has also been detected in MGUS, its prevalence is much higher in advanced MM and extramedullary forms of the disease.

Prognostic significance of chromosomal aberrations in myeloma

Gene expression analysis of MM has confirmed the huge genetic diversity of this malignancy. Recently, the classification of MM into seven different groups has been proposed. Each group displays a specific genetic signature and some of them are associated with a particular *IGH* translocation or ploidy status and with a characteristic clinical behaviour. Table 28.1 summarises this classification, which connects genetic abnormalities, cell transcriptome and clinical features of patients.

Dysregulation of cyclin D genes as a potential unifying event in MM pathogenesis

There is no common genetic mechanism to explain the pathogenesis of MM. However, it can be speculated that although *IGH* translocations induce upregulation of different oncogenes, it is possible that all *IGH* translocations involved in MM converge on a common pathway resulting in inhibition of differentiation and an increase in cell survival and proliferation. Gene expression profiling analysis has demonstrated that expression of *CCND1*, *CCND2* and *CCND3* is increased in virtually all MM patients, supporting the recent hypothesis of a potential unifying event in pathogenesis. Approximately 25% of MM cases display over-expression of one of these cyclins, which may be triggered directly by an *IGH* translocation such as t(11;14) and t(6;14) that dysregulates *CCND1* and *CCND3* respectively, or indirectly by an *IGH* translocation involving *MAF* and *MAFB* genes, which encode a transcription factor that targets cyclin D2. Nearly 40% of MM cases express increased cyclin D1 through biallelic dysregulation of *CCND1* and without apparent t(11;14); most of the remaining cases of MM, including those with t(4;14), have increased expression of cyclin D2.

MicroRNA expression

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level and are involved in critical biological processes, including cellular growth and differentiation. Different studies have shown that miRNA expression is deregulated in myeloma cells as compared to normal plasma cells and that their expression profile is associated with genetic abnormalities. Moreover, several miRNAs have been involved in MM pathogenesis. In this sense, it has shown a mechanism of p53 regulation through miRNAs acting on *MDM2* expression; thus, miR-192, 194 and 215 re-expression in myeloma cell lines induce degradation of *MDM2* with the subsequent p53 upregulation and cell growth inhibition.

Multistep pathogenesis of multiple myeloma

The current pathogenic models assume that MM develops through a multistep transformation from normal plasma cells (PCs) to MGUS, which implies PC immortalisation and, subsequently, the transformation to active MM, where clonal PCs cause end-organ damage. Cytogenetic studies using FISH have demonstrated that most genetic lesions typical of MM are already present at MGUS stage. Recent studies show that a major difference between these three entities is the number of PCs with genetic abnormalities, which increases from MGUS to SMM (smouldering multiple myeloma) and to MM, thus the progression from MGUS to SMM, and eventually to MM, involves a clonal expansion of genetically abnormal PCs. These findings have also been confirmed WGS. WGS in longitudinal samples has shown evidence for Darwinian evolution with both linear and branching sub clonal changes in patients with some additional spatio temporal variation. In addition, contribution from parallel changes in the immune microenvironment during this process is an active area of investigation. Ability of clonal plasma cells to evade immune surveillance and enrichment of the marrow with pro-tumoural immune microenvironment (Higher T regulatory cell numbers, T cell exhaustion, increased in myeloid-derived suppressor cells) support progression from MGUS to myeloma.

Interaction between plasma cells and their microenvironment

As far as the pathogenesis of MM is concerned, interactions between the myelomatous plasma cells and their resident marrow microenvironment have been studied in detail. In the bone marrow, MM cells adhere to extracellular matrix proteins and bone marrow stromal cells through a series of adhesion molecules, for example the β_1 integrin family (VLA-4, VLA-5, VLA-6; also called CD49d, CD49e and CD49f) (present in myeloma cells), and vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (present in stromal cells) (Figure 28.2). In addition, bone

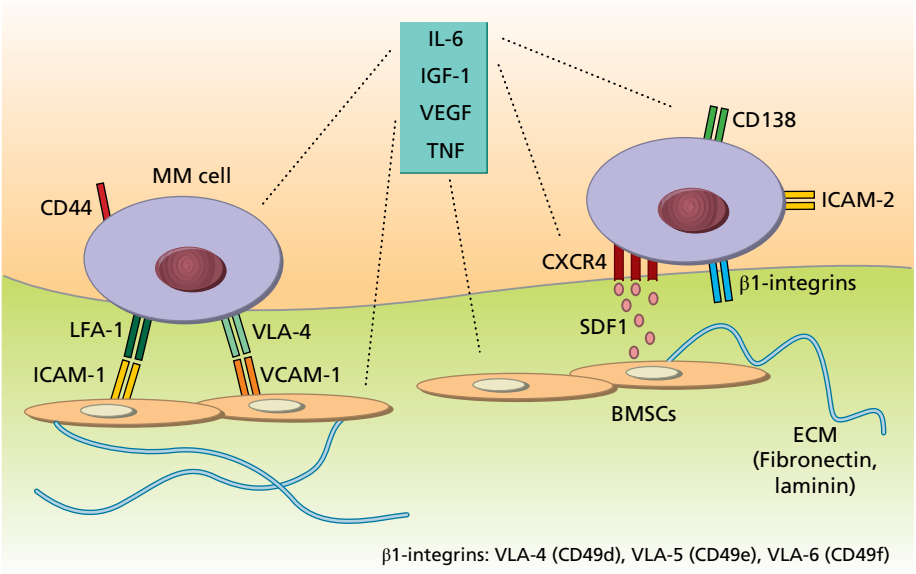

Figure 28.2 Interactions between plasma cells and the microenvironment. See text for definition of abbreviations.

marrow stromal cells produce a stromal-cell-derived factor (SDF)-1 that binds to CXCR4 on the surface of myeloma cells, inducing both chemotaxis of plasma cells and upregulation of surface adhesion molecules such as VLA-4. Bone marrow homing of plasma cells is likely further facilitated through other adhesion molecules expressed by myeloma cells, such as CD138, CD38, CD44 and CD106. Interaction of CD147 receptor on myeloma cells with cyclophilin-A secreted by bone marrow endothelial cells also promotes plasma cell homing. The binding of MM cells to the bone marrow stromal cells induces the transcription and secretion of cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, insulin-like growth factor (IGF)-1, IL-21, SDF-1α and vascular endothelial growth factor (VEGF), by plasma cells and/or bone marrow stromal cells; this trigger signalling pathways (e.g. RAF/MEK/MAPK, PI3K/AKT, NF-κB and JAK/STAT) that promote plasma cell proliferation and prevent apoptosis. These pathways are also potential targets for therapeutic intervention (Figure 28.3). In addition, cytokines modulate the production of additional adhesion molecules, which, in a vicious circle, enhances cell adhesion.

Adhesion of myeloma cells to the bone marrow microenvironment induces a cell adhesion-mediated drug resistance phenotype via three mechanisms: (i) cell cycle arrest at G_1 (associated with upregulation of p27, an inhibitor of cyclin-dependent kinases), (ii) apoptosis inhibition via over-expression of FLIP-L, an endogenous inhibitor of FAS (CD95) and (iii) protection of tumour cells from initial drug-induced DNA damage (double-strand breaks) by reducing topoisomerase II activity(iV) enhancing Nfkb activity.

In summary, it appears that the bone marrow microenvironment provides a sanctuary for myeloma cells by both promoting proliferation and blocking apoptosis, thereby allowing tumour

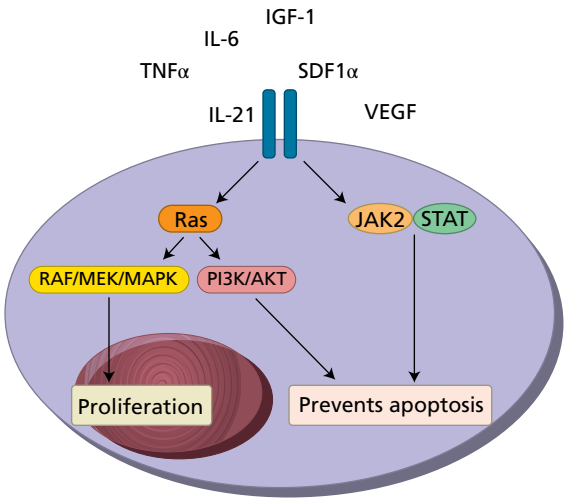

Figure 28.3 Signalling pathways involved in myeloma pathogenesis. See text for definition of abbreviations.

survival/progression and eventual emergence of drug resistance. Interruption by downregulating the interactions between the tumour cell and its marrow microenvironment can potentially halt cell growth and proliferation and be therapeutically value.

Influence of pathogenesis on the clinical features of MM and the development of bone lesions

The interaction between MM cells and bone marrow microenvironment not only favours tumour growth but is also responsible for the dominant clinical feature of myeloma bone disease (see below and Figure 28.4). This interaction stimulates other

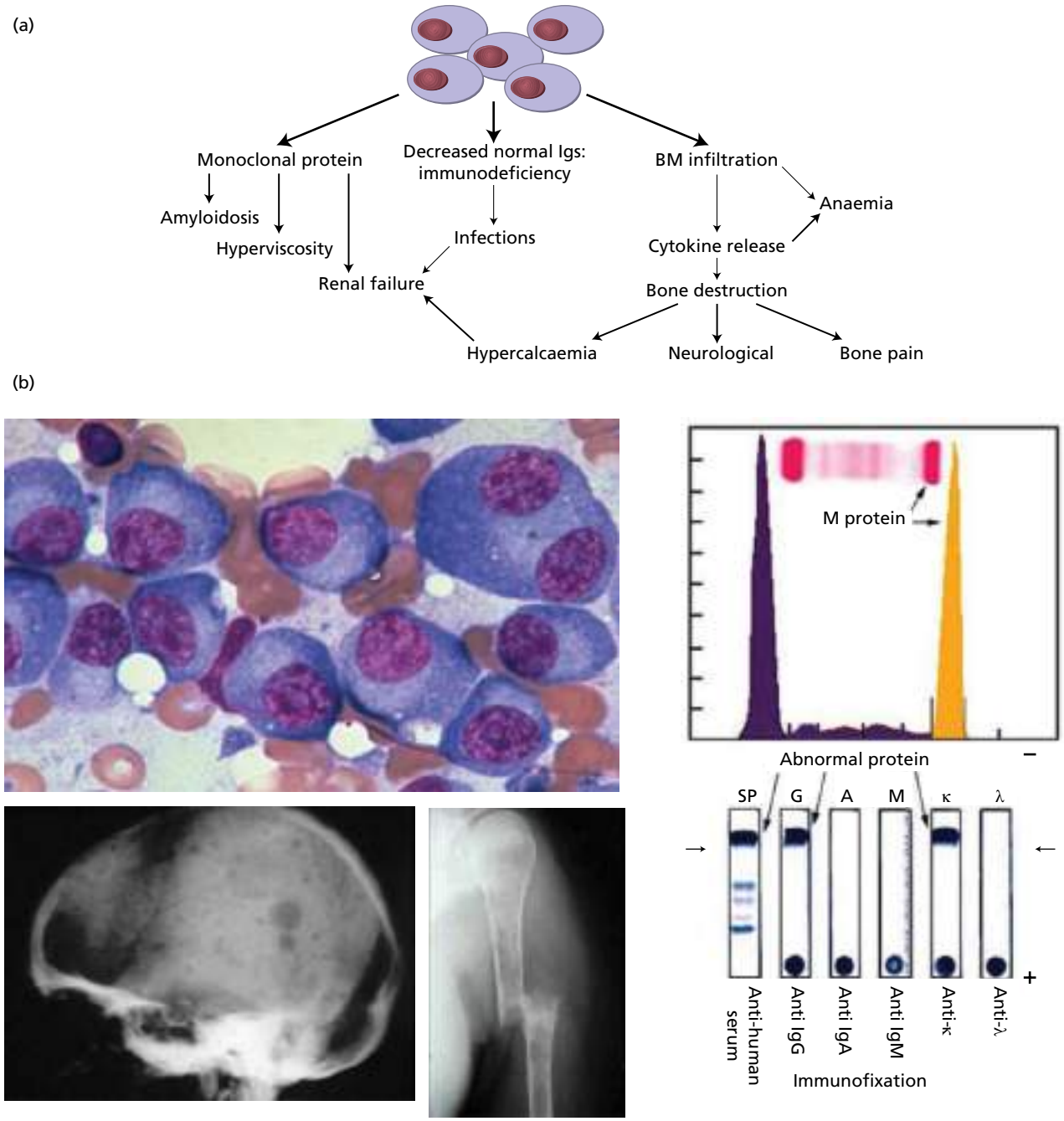

Figure 28.4 (a) Clinical manifestations in myeloma and (b) bone marrow infiltration, monoclonal band (IgGK by immunofixation) in serum, osteolytic lesions resulting in a pathological fracture.

cytokine cascades responsible for osteolytic lesions, which can result in bone pain, hypercalcaemia and neurological compression syndromes. Bone marrow infiltration with MM cells also impairs normal haematopoiesis, leading to anaemia. MM cells secrete monoclonal protein (M-protein) that increases plasma viscosity. In addition, M-protein, and particularly light chains, are responsible for the impairment of renal function, leading ultimately to renal failure. Hypercalcaemia due to osteolytic disease may also contribute to renal failure. Patients are also at increased risk of developing potentially life-threatening infections due to the lack of functional immunoglobulins.

Bone disease is one of the hallmarks of MM (see below). Osteolysis is mediated by an imbalance between osteoclast

activity (increased) and osteoblast activity (decreased). Interaction of MM cells with stromal cells and other cells (including T cells) in the microenvironment induces the secretion of numerous osteoclast-activating factors such as RANK-L, macrophage inflammatory protein (MIP)-1α (also known as CCL3), activin A, VEGF, hepatocyte growth factor (HGF), IL-3, IL-7, TNF-α, IL-6, IL-1β and MIP-3α. Two of the most important are RANK-L (receptor activator of NF-κB ligand) and MIP-1α.

RANK-L is a transmembrane molecule in stromal cells/osteoprogenitors, which is also called TRANCE (TNF-related activation-induced cytokine) or OPG-L (osteoprotegerin ligand). RANK-L binds to its functional receptor RANK (TNF receptor superfamily) on osteoclasts, stimulating osteoclastogenesis by inducing differentiation of osteoclast precursors and stimulating resorption. RANKL activity can be blocked by osteoprotegerin (OPG), a soluble-decoy receptor for RANK-L also produced by stromal/osteoprogenitor cells. Therefore, osteoclastic activity is regulated by a delicate balance between RANK-L and OPG. In fact, under normal physiological conditions, the levels of OPG are significantly higher than those of RANK-L. In contrast, in MM this balance is disrupted by increased expression of RANK-L and decreased expression of OPG on stromal cells after interaction with myeloma cells. It has been suggested that the decline in OPG is also mediated by myeloma cell uptake by binding to CD138 and subsequent degradation. MIP-1α is a potent stimulator of osteoclast formation through a dual mechanism: (i) it enhances the activity of RANK-L and (ii) it directly stimulates osteoclast precursors to differentiate into mature forms. MIP-1α gene expression is abnormally regulated in MM due to unbalanced expression of the acute myeloid leukaemia (AML)-1A and AML-1B transcription factors in myeloma cells. This imbalance also induces IL-3, which stimulates osteoclast formation and resorption directly or by further augmenting that of RANKL and MIP-1α; in addition, as mentioned below, IL-3 inhibits osteoblast formation. Other chemokines such as IL-7, TNFα and IL1β indirectly stimulate osteolytic processes, inducing RANK-L expression. Other OAFs secreted by myeloma cells and/or stromal/or osteoclast cells (e.g. HGF, IL-6, VEGF, activin A), further increase the gradient of osteoclastogenic factors in focal lesions and contribute to osteoclast production and activity.

In MM, in addition to the marked osteoclast activation, there is inhibition of osteoblast formation and function, which is mediated by both soluble factors and direct cellular interactions of myeloma and stromal cells. The WNT/bone morphogenetic protein (BMP) signalling pathways are critical for the osteogenic differentiation of mesenchymal stem cells to mature bone-forming osteoblasts. Myeloma cells (and other BM microenvironmental cells) produce numerous soluble factors that inhibit osteoblast differentiation and/or function, such as WNT signalling antagonists (e.g. DKK1, sclerostin, soluble frizzled related proteins (sFRP-2/3)), BMP inhibitors (e.g. activin A, TGF-β and HGF), and other cytokines and chemokines (e.g. IL-7, TNF-α and IL-3 (which indirectly inhibits osteoblast differentiation involving CD45+ cells in the BM)). In addition, osteoblastic cells from myeloma patients with lytic lesions show reduced Runx2 activity, Runx2 being the major transcription factor regulating osteoblast differentiation. Suppression of Runx2 activity is mediated, at least in part, by cell-to-cell contact of myeloma and mesenchymal osteoprogenitors, since blocking of VLA4-VCAM1 interactions with a neutralising anti-VLA4 antibody partially restored Runx2 function in mesenchymal cells.

Differential diagnosis

The diagnostic criteria for the monoclonal gammopathies have been reviewed by the International Myeloma Working Group (IMWG). The main clinical entities are MGUS, primary systemic amyloidosis (see also Chapter 29), smouldering multiple myeloma and symptomatic multiple myeloma.

Monoclonal gammopathy of undetermined significance

MGUS has a high prevalence (3.2 and 5.8% in individuals over 50 and 70 years of age, respectively). It is characterised by the presence of a serum M-protein (<30 g/L) and less than 10% plasma cells in the bone marrow with no evidence of other B-cell lymphoproliferative disorder and no symptoms or organ or tissue impairment due to the monoclonal gammopathy. There are three distinct clinical types of MGUS based on the secreted monoclonal protein. They are seen distinctly as the progressed malignant phenotype can be different (myeloma or Waldenstroms macroglobulinaemia) and rates of progression also differ (Table 28.2). The transformation rate to a malignant plasma cell disorder is about 1% per year, with an actuarial probability of malignant evolution of 30% at 25 years of follow-up. When the different causes of death are considered, the actuarial probability of malignant transformation at 25 years of follow-up is only 11%, much lower than the actuarial prediction. The main factors associated with MGUS progression include M-protein size, IgA isotype, abnormal free light-chain ratio and the 'evolving type' (rising M-protein during the first years of follow-up), and the presence of more than 95% phenotypically aberrant plasma cells within the bone marrow compartment. Utilising blood-based risk factors, M protein size >15 g/L; non-immunoglobulin G isotype; abnormal free light chain ration <0.26 or > 1.65, the risk of progression to malignant disease at 20 years (adjusted for competing causes of death) is 2, 10, 18 and 27% based on presence of 0, 1, 2 or 3 risk factors.

Table 28.2 Types of monoclonal gammopathy of undetermined significance.

	IgM MGUS	Non-IgM MGUS	Light-chain MGUS
M protein	Presence of serum IgM (<30.0 g/L)	Presence of serum IgA or IgG (<30.0 g/L)	No heavy-chain Ig Abnormal FLC ratio (<0.26 or > 1.65)*
BM involvement	Lymphoplasmacytic infiltration <10%	Clonal BM plasma cells <10%	Clonal BM plasma cells <10%
End organ damage	None†	None‡	None‡
Potential to progress to (lifetime risk/year)	Waldenstorms's macroglobulinaemia, NHL, or AL amyloidosis (1.4%) IgM myeloma (very rare)	Myeloma or a lymphoproliferative disorder (1%)	Light-chain myeloma (0.4%)

AL, amyloid light-chain [amyloidosis]; BM, bone marrow, FLC, free light chain; Ig, immunoglobulin; MGUS, monoclonal gammopathy of undermined significance; NHL, non-Hodgkin lymphoma.
* sFLC ratios increase with declining renal function. Renal reference range ratio of 0.26–3.1 applies here for patients with chronic kidney disease stages 3–5.
† No evidence of anaemia, constitutional symptoms, hyperviscosity, lymphadenopathy, hepatosplenomegaly, or other end organ damage that can be attributed to the underlying lymphoproliferative disorder.
‡ Absence of end organ damage based on CRAB criteria ([hyper] Calcemia, Renal insufficiency, Anaemia, Bone lesions) or amyloidosis that can be attributed to the plasma cell proliferative disorder.

Monoclonal gammopathy of clinical significance (MGCS)

Although MGUS may not transform to myeloma in vast majority of patients, the monoclonal protein secreted can cause organ damage directly or through other mechanisms. This has now been increasingly recognised in clinical practice, although the prevalence of these pathologies remains unclear. A well characterised Monoclonal gammopathy of clinical significance (MGCS) condition is Amyloidosis discussed in Chapter 29. Another group of disorders affecting renal parenchyma are grouped under monoclonal gammopathy of renal significance (MGRS). The monoclonal protein can directly injure renal tissue or mediate damage through autoantibody activity. Skin disorders, neuropathy, systemic disorders such as cryoglobulinaemia, C1 inhibitor deficiency, late-onset nemaline myopathy and capillary leak syndrome are other distinct clinical presentations aetiologically related to the presence of monoclonal protein. Treatment is targeted to underlying abnormal clone of either abnormal B cells or plasma cells.

Smouldering multiple myeloma

The term 'smouldering multiple myeloma' (SMM) was first defined by Kyle and Greipp as the presence of a serum M-protein (>30 g/L) and 10% or more plasma cells in the bone marrow in the absence of lytic bone lesions or clinical manifestations due to the monoclonal gammopathy. More recently, the IMWG considered that the term 'asymptomatic myeloma' could be more appropriate. This condition was defined as the presence of an M-protein (≥30 g/L) and/or 10% or greater bone marrow plasma cells in the absence of symptoms or organ or tissue impairment due to the monoclonal gammopathy. About 10% of patients diagnosed with MM have smouldering disease. This situation is clinically and biologically very close to that observed in MGUS. However, the plasma cell mass is much higher, and most cases will eventually evolve into symptomatic MM.

The annual risk of progression to symptomatic disease is 10% per year for the first 5 years, and it significantly decreases thereafter, 5% per year during the following 5 years and only 1% per year from the 10th year. SMM is not a uniform entity, since it ranges from indolent, low-risk forms (that behave as MGUS) to 'early myelomas' at high risk of developing symptomatic disease. This heterogeneous outcome is supported by the identification of risk factors predicting progression to symptomatic MM. The Old Mayo Clinic Risk Classification proposed three different subgroups of SMM: group 1 with ≥3 g/dL of monoclonal protein and ≥10% of plasma cells in bone marrow, in which the median time to progression (TTP) to symptomatic MM is 2 years; group 2 with <3 g/dL of monoclonal protein and ≥10% bone marrow plasma cells M-protein with a median TTP of 8 years and group 3 with ≥3 g/dL of monoclonal protein, but with <10% plasma cells bone marrow infiltration, translating into a median TTP of 19 years. The Spanish group has proposed a risk classification based on the percentage of PCs with aberrant phenotype (high risk if ≥95% of the total PCs are clonal) plus immunoparesis (decrease in one or two of the uninvolved immunoglobulins), with a median TTP of 23 months when the two risk factors were present, as compared with 73 months when only one risk factor was present and not reached when

none of the risk factors was present. Other adverse risk factors include an abnormal serum free light chain (sFLC), evolving levels of paraprotein and cytogenetic abnormalities such as t(4;14), gain of 1q21 or hypodiploidy. Revised Mayo clinic risk classification has proposed 3 different groups based on 3 variables; bone marrow plasma cells >20%, involved to uninvolved sFLC ratio > 20, >2 g/dL of monoclonal protein. The 2 year progression risk: 6% for low risk (no risk factors), 18% for intermediate risk (1 factor) and 44% for high risk (2–3 factors). These patients should be considered for early intervention in the context of clinical trials. These risk stratification classifications may allow a more individualised disease management.

Multiple myeloma

The diagnosis of MM requires the presence of an M-protein in serum and/or urine, increased plasma cells in the bone marrow or plasmacytoma and related organ or tissue impairment (including bone lesions). The more common symptoms are fatigue from anaemia and bone pain due to the skeletal involvement. Some patients may have no symptoms, but they can have related organ or tissue impairment. Clinical and laboratory features may include anaemia, skeletal involvement (lytic lesions and/or severe osteoporosis, with or without compression fractures), renal failure, hypercalcaemia, recurrent bacterial infections, extramedullary plasmacytomas or associated amyloidosis (Table 28.3). The criteria revised by the IMWG for the diagnosis of MM now includes biomarkers of malignancy which are: more than 60% plasma cells, involved: uninvolved sFLC ratio of over 100 and more than one focal lesion on MRI studies of 5 mm of more in size, shown in Table 28.4. Of note, no serum or urine M-protein values were included, since about 40% of patients with symptomatic MM have a serum M-protein level lower than 30 g/L and 3% have non-secretory myeloma. In the same sense, no minimal proportion of bone marrow plasma cells was required because about 5% of patients with well-documented symptomatic MM have less than 10% plasma cells in their bone marrow. Table 28.5 illustrates the work-up for patients with monoclonal gammopathies.

Table 28.3 Myeloma-related organ or tissue impairment (end-organ damage) due to the plasma cell proliferative process.

- Increased serum calcium
- Renal insufficiency
- Anaemia: haemoglobin 20 g/L below the lowest normal limit
- Bone lesions: lytic lesions or osteoporosis with compression fractures (possibly confirmed by MRI or CT)
- Other: symptomatic hyperviscosity (rare), amyloidosis, recurrent bacterial infections (more than two episodes in 12 months), extramedullary plasmacytomas

Table 28.4 Diagnostic criteria for Multiple myeloma. Clonal bone marrow plasma cells ≥10%, or biopsy proven plasmacytoma and any one of myeloma defining events or biomarkers of malignancy.

- Hypercalcaemia >2.75 mmol/L
- Renal insufficiency creatinine clearance <40 mL/min
- Anaemia, haemoglobin <100 g/L
- Bone lesions one or more osteolytic lesions on imaging
- Clonal bone marrow plasma cells ≥60%
- Involved: uninvolved serum free light chain ratio ≥100
- >1 focal lesions on MRI studies

Table 28.5 Work-up for a patient with monoclonal gammopathy.

- History and physical examination
- Complete blood count and differential peripheral blood film
- Chemistry including calcium and creatinine
- Serum protein electrophoresis and immunofixation
- Nephelometric quantification of immunoglobulins
- 24-hour urine collection for electrophoresis and immunofixation
- Bone marrow aspirate (Morphology, Myeloma FISH in CD138 selected cells and immunophenotyping)
- Radiological skeletal bone survey: whole body CT, whole body MRI or PET.CT
- β_2-Microglobulin, C-reactive protein and lactate dehydrogenase
- Serum free monoclonal light chains

Other special forms of plasma cell dyscrasia

Plasma cell leukaemia

Plasma cell leukaemia was initially described by Kyle in 1974 as a plasma cell disorder characterised by a relative peripheral blood plasmacytosis of more than 20% of total nucleated cells, or an absolute number of plasma cells greater than 2×10^9/L. It is likely that lower levels of circulating plasma cells (i.e. ≥5% and/or $\geq 500 \times 10^9$/L has a similar prognostic meaning. There are two forms of plasma cell leukaemia: the *de novo* presentation in leukaemic phase, and secondary cases corresponding to already diagnosed MM that evolve into a leukaemic phase. The clinical course of plasma cell leukaemia is usually very aggressive and resistant to conventional treatment and therefore new agents should be urgently investigated in these patients.

Solitary plasmacytoma of bone

The existence of a solitary plasmacytoma has been recognised in up to 3% of patients with a plasma cell dyscrasia, usually on the vertebral column. The diagnostic criteria require the existence

of a solitary plasma cell tumour in which the biopsy confirms plasma cell histology, a negative skeletal survey and absence of plasma cell infiltration in a random bone sample (<10%), as well as no evidence of anaemia, hypercalcaemia or renal impairment. Some groups suggest that patients in whom a paraprotein persists after the eradication of plasmacytoma with local treatment should undergo a review of the diagnosis. The treatment of choice is local radical curative radiotherapy, but about two-thirds of patients with solitary bone plasmacytoma develop MM at 10 years' follow-up, with a median time to progression of 2 years.

Extramedullary plasmacytoma

Extramedullary plasmacytoma is a plasma cell tumour that arises outside the bone marrow, most frequently in the upper respiratory tract (nose, paranasal sinuses, nasopharynx and tonsils). Other sites include parathyroid gland, orbit, lung, spleen, gastrointestinal tract, testes and skin. In most cases the lesion is unique, although the presence of more lesions (multiple plasmacytomas) has also been reported. Diagnosis is based on the detection of the plasma cell tumour in an extramedullary site, in the absence of bone marrow plasma cell infiltration, bone lytic lesions and other signs of MM (end-organ damage) Treatment involves, plasmacytoma resection and/or radiotherapy. Large or resistant plasmacytomas may require systemic therapy.

Non-secretory multiple myeloma

This specific type of MM requires particular attention, since it is very difficult to diagnose. The only way to make a definitive diagnosis is to demonstrate the presence of tissue infiltration (usually bone marrow) by cells with plasma cell morphology. However, plasma cell infiltration must be greater than 10% and clonality must be assessed by immunophenotyping (demonstration of cytoplasmic immunoglobulins with restricted light chain: positive production without excretion). In addition, the serum free light chains are abnormal often with positive imaging findings (FDG avidity) and these are useful parameter for the follow-up. However, exceptional cases exist in which no monoclonal protein can be observed within the plasma cells. In these cases, it is mandatory to demonstrate clonality by the study of the rearrangement status of the immunoglobulin genes.

IgM multiple myeloma

This exceptional form of myeloma has been reported very rarely and must be distinguished from Waldenström macroglobulinaemia. The morphology and immunophenotype of the infiltrating cells will give the definitive diagnosis, as well as the existence of osteolytic lesions on imaging, which are typically absent in Waldenström macroglobulinaemia. Recent studies have shown IgM myeloma developing from pre germinal centre B cells; up to 60% of patients have t(11;14) and proportion of patients with chromosome 6 and 13 deletion. Cells have a high expression of CD20 and bcl2.

Osteosclerotic myeloma (POEMS syndrome)

POEMS syndrome is characterised by polyneuropathy, organomegaly, endocrinopathy, M-protein and skin changes. The clinical picture consists of a chronic inflammatory demyelinating polyneuropathy, more motor than sensory, and osteosclerotic lesions. Hepatomegaly, hyperpigmentation, hypertrichosis, angiomatous lesions on the trunk, gynaecomastia, testicular atrophy and papilloedema or thrombocytosis may occur. The M-protein is commonly of IgA λ type and the bone marrow contains less than 5% plasma cells. Castleman disease can be associated and VEGF is universally increased. Biopsy of an osteosclerotic lesion may be necessary to confirm the diagnosis. Mandatory diagnostic criteria are the presence of an M-protein and polyneuropathy, and major criteria are the presence of osteosclerotic lesions, Castleman disease and elevated VEGF. At least, the two major criteria plus one major finding and a minor clinical criteria (above mentioned) are necessary for diagnosis.

Amyloid disease is dealt with in Chapter 29.

Disease complications and their management

Figure 28.4 illustrates the clinical manifestations of multiple myeloma.

Bone involvement: assessment and treatment

Bone involvement is the most frequent clinical complication in patients with MM. About 70% of patients have lytic bone lesions, with or without osteoporosis, and another 20% have severe osteoporosis without lytic lesions. This frequency corresponds to conventional skeletal radiography assessment, a technique that is associated with low sensitivity (only demonstrating lytic disease when at least 30% of bone substance has been lost), particularly in some areas (ribs, sternum), low specificity (gas in colon) and long examination time. Newer imaging techniques have greater sensitivity compared with radiographic bone survey for detection of MM bone lesions (Figure 28.5). Computed tomography (CT) has the highest sensitivity for the detection of bone defects and with the whole-body low-dose modality the radiation exposure is much lower than with conventional CT, the scanning time is short and it may replace conventional X-ray in the near future. Magnetic resonance imaging has the highest resolution for soft tissue and bone marrow infiltration; it is particularly valuable for differentiation between benign and malignant fractures, but is inferior to CT for assessment of bone disease. Finally, positron emission tomography (PET) allows

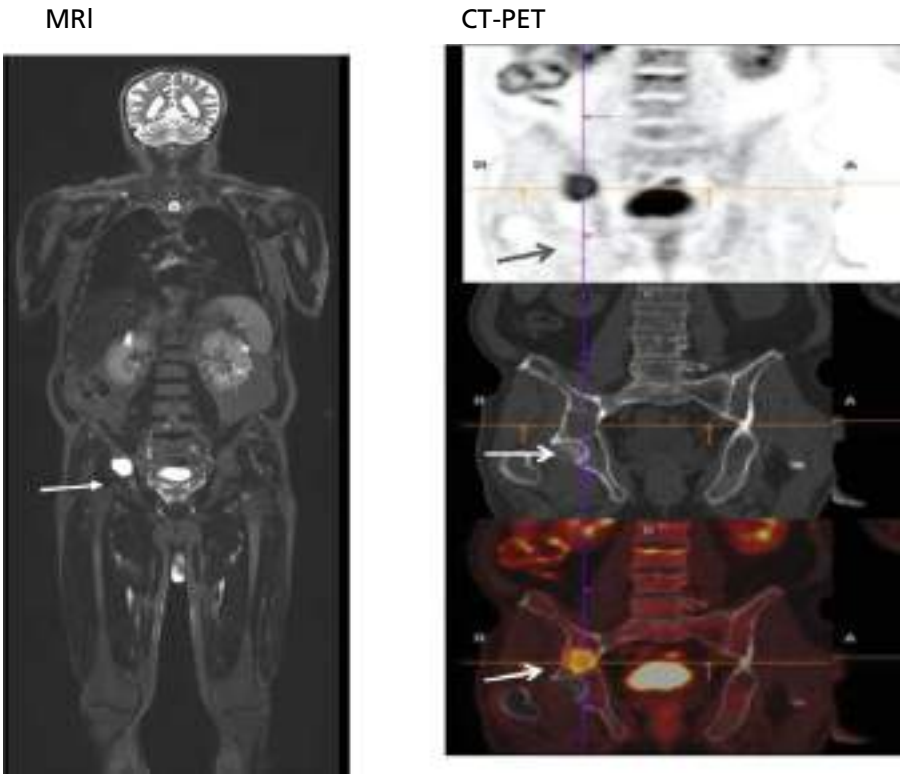

Figure 28.5 MRI and PET/CT in a myeloma patient. The arrows show a myeloma lesion in the lower part of the right iliac bone. The T2 weighted MRI shows a hyperintense image (left) and an active lesion at FDG PET/CT is confirmed (right).

assessment of tumour metabolism and disease activity (versus inactive or necrosis), and may be of prognostic significance; however, it still requires work on standardisation.

These new techniques are already recommended to evaluate patients with SMM and solitary plasmacytoma, and although up until now the presence of one or more clear sites of osteolytic bone destruction seen on CT and/or PET-CT does fulfil the diagnostic criteria, the situation will probably change soon. Nevertheless, great caution should be paid to avoid over-interpretation of equivocal or tiny lucencies seen only on CT or PET-CT.

From the clinical point of view, skeletal involvement leads to bone pain and can result in pathological fractures. The pathophysiology of bone disease has been described above and is summarised in Figure 28.6. Some patients develop pathological fractures of long bones and require orthopaedic surgery. In the event of extensive lesions, surgery can be followed by radiation therapy. On the other hand, prophylactic orthopaedic intervention must be considered in patients with large lytic lesions at high risk of fracture. It is important to consider that patients with severe back pain due to vertebral compression fractures can benefit from vertebroplasty or kyphoplasty. Spinal cord compression caused by a vertebral fracture is very rare in patients with MM. This complication is usually caused by a plasmacytoma arising from a vertebral body.

Between 15 and 20% of patients with MM have hypercalcaemia at the time of diagnosis. A common complication of hypercalcaemia is renal impairment caused by interstitial nephritis. Treatment of hypercalcaemia with hydration and bisphosphonates is a medical emergency. Zoledronic acid is the bisphosphonate of choice (quicker response and significantly longer time to recurrence compared with pamidronate).

The intravenous agents pamidronate and zoledronic acid are of clinical benefit in the treatment of bone disease in patients with MM. Pamidronate is administered at a monthly dose of 90 mg via a 2-hour intravenous infusion. Zoledronic acid, at a monthly dose of 4 mg, is at least as effective as pamidronate and has the advantage that it can be administered via a 15-min infusion. In patients with renal function impairment, the dose of zoledronic acid must be reduced to a maximum of 3 mg. It was suggested that bisphosphonates should be used indefinitely, once initiated. However, the appearance of severe late complications, such as osteonecrosis of the jaw, related to the duration of bisphosphonate exposure has resulted in a reconsideration of the initial recommendations. Osteonecrosis of the jaw is associated with the duration of bisphosphonate exposure, type of bisphosphonate (higher with zoledronic acid than with pamidronate) and history of recent dental procedure. The current recommendations for treatment with bisphosphonates in MM patients, based on consensus panels from both the IMWG and the ASCO, do not recommend the initial use of bisphosphonates for more than 2 years. Finally, in renal impaired patients, the recently

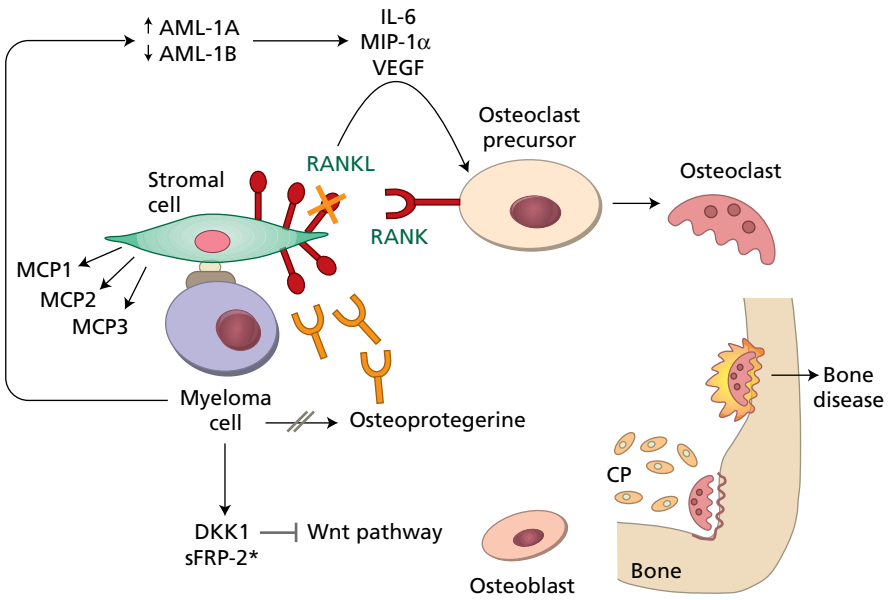

Figure 28.6 Pathogenesis of bone disease. See text for definition of abbreviations.

approved agent denosumab might be of benefit. Denosumab was shown to be non-inferior to Zoledronic acid. In relapsed patients, treatment with bisphosphonates or Denosumab can be re-started and administered concomitantly with active therapy.

Renal failure

About 20% of patients with MM have a serum creatinine higher than 177 µmol/L (2 mg/dL) at diagnosis. The degree of renal failure is usually moderate, with a serum creatinine lower than 354 µmol/L (4 mg/dL). However, in some series up to 10% of patients with newly diagnosed MM have renal failure severe enough to require dialysis from the time of diagnosis. The main causes of renal failure in MM are: (i) light-chain excretion resulting in cast nephropathy (myeloma kidney) and (ii) glomerular deposition of immunoglobulin (light-chain amyloidosis or immunoglobulin deposition disease). In myeloma kidney, the typical feature consists of the presence of myeloma casts, mainly composed of light chains, in the distal tubules and collecting ducts. There is a correlation between the degree of cast formation and the severity of renal failure. Light-chain tissue deposition usually consists of glomerular deposits of immunoglobulins, resulting in nephrotic syndrome. The amyloid deposits are fibrillar structures of light chains showing positive Congo red staining. In light-chain deposition disease, the deposit of light-chain immunoglobulins is non-fibrillar (Congo red negative). In contrast with amyloidosis, the light chain is usually of the κ type. The characteristic clinical feature is a nephrotic syndrome, but renal function can rapidly deteriorate, resembling glomerulonephritis (see also Chapter 29).

The median survival of patients with MM and renal insufficiency is significantly lower than patients with normal renal function. However, the prognosis mainly depends on the reversibility of renal function. Thus, the median survival of patients with reversible renal failure is similar to that of patients with normal renal function, whereas patients with non-reversible renal failure have a median survival of a few years. The factors associated with renal function recovery include serum creatinine lower than 354 µmol/L (4 mg/dL), 24-hour urinary protein excretion lower than 1 g, and serum calcium higher than 2.875 mmol/L (11.5 mg/dL).

The mechanism of action of bortezomib is rapid, and it is probably an ideal agent for rapidly decreasing light chains in order to prevent the development of irreversible renal failure by avoiding further tubular light-chain damage. In a retrospective series of 24 patients with relapsed/refractory MM and dialysis-dependent renal failure, the overall response rate (RR) was 75% with 30% complete remissions (CR) or near-CR. Recent studies have confirmed the benefit of bortezomib-based therapies in patients with newly diagnosed myeloma and renal failure. Bortezomib based combinations should be considered to deepen remissions and induce renal reversibility.

With regard to the use of high-dose therapy/autologous stem cell transplantation (SCT) in patients with MM and renal failure, the largest experience comes from the Arkansas group, with a reversibility of renal failure of 43%, but higher morbidity and mortality (6 and 13% after a single or tandem transplant, respectively) than in patients with normal renal function. Chemo resistant disease, low serum albumin and older age were associated with a poorer outcome. In any event, the dose of melphalan must be reduced to 140 mg/m^2. In patients with no overt myeloma and low plasma cell mass

in whom renal function impairment is due to glomerular light-chain deposition (light-chain deposition disease), the likelihood of response is higher than that in MM because of the low plasma cell mass at the time of transplantation. In this situation there is no need for tumour reduction with induction chemotherapy before stem cell mobilisation and high-dose therapy.

Theoretically, the removal of nephrotoxic light chains with plasma exchange could avoid further renal failure and hopefully prevent irreversible renal failure. MYRE and Eulite randomised trials failed to conclusively demonstrate that use of high cut-off dialysis results in higher proportion of patients recovering kidney function. Therefore, early diagnosis, preventing nephrotoxic agents, hydration and use of effective antimyeloma therapies are the measures to improve renal function in these patients

Anaemia and bone marrow failure

Approximately 35% of patients with newly diagnosed MM have a haemoglobin level lower than 90 g/L. In addition, severe anaemia is a frequent complication later in the course of the disease due to disease progression. Anaemia is associated with a significant loss in quality of life and poor prognosis. The main causes of anaemia in MM are bone marrow replacement by plasma cells, relative erythropoietin deficiency, renal insufficiency and chemotherapy with cytotoxic agents.

Severe granulocytopenia and thrombocytopenia at the time of diagnosis are unusual. About 10% of patients have a platelet count of less than 100×10^9/L, but platelet counts lower than 20×10^9/L with risk of severe bleeding are very unusual. The development of an unexplained pancytopenia in patients previously treated with alkylating agents, particularly melphalan, is suspicious of myelodysplasia.

A number of trials have shown the beneficial effect of recombinant human erythropoietins and darbepoetin-α in the treatment of myeloma-associated anaemia. The response to erythropoietin is associated with a significant improvement in quality of life. An important aspect is that the most significant improvement in quality of life is reached when the haemoglobin increases from 110 to 120 g/L, but levels above 140 g/L should be avoided due to its association with a higher risk of thrombosis. Thus, the goal should be maintenance of the haemoglobin around 120 g/L, with careful dose titration in order to achieve a good quality of life, while minimising severe complications such as thrombotic events. The major cause of erythropoietin failure is iron deficiency. Iron repletion should be indicated when there is evidence of functional iron deficiency measured by an increased soluble transferrin receptor. It seems that the best iron supplemental therapy is the administration of iron saccharate. Treatment with granulocyte colony-stimulating factor (G-CSF) may be required to treat chemotherapy-induced severe granulocytopenia.

Infection

Infectious complications are the major cause of morbidity and mortality in patients with MM. The highest risk of infection is observed during the first 2 months of starting therapy, in patients with severe chemotherapy-induced granulocytopenia and in those with relapsed and refractory disease. The main cause of infection in MM is the impaired antibody production, leading to a decrease in the uninvolved immunoglobulins. Other important causes include chemotherapy-induced granulocytopenia, renal function impairment and glucocorticoid treatment, particularly high-dose dexamethasone. Most infections in newly diagnosed patients and during the first cycles of chemotherapy are caused by *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae*, while in patients with renal failure, as well as in those with relapsed and/or refractory advanced disease, more than 90% of the infectious episodes are caused by Gram-negative bacilli or *Staph. aureus*. The TEAMM trial tested use of fluroquinolones for a 3-month period for newly diagnosed myeloma patients. Use of fluroquinolones reduced the number of febrile episodes and deaths without inducing increase in healthcare associated infections.

An infectious episode in a patient with MM should be managed as a potentially serious complication requiring immediate therapy. In case of suspected severe infection and before the identification of the causal agent, treatment against encapsulated bacteria and Gram-negative microorganisms should be initiated. Routine use of immunoglobulin prophylaxis is not usually recommended. But select patients may benefit from this approach. Pneumococcal vaccination is recommended, particularly in patients with IgG myeloma with high-serum M-protein levels, which are usually associated with very low levels of uninvolved immunoglobulins. Patients should receive prophylaxis against varicella-zoster infections and should be vaccinated against shingles using Shingrix recombinant vaccination. Annual flu vaccination is also recommended. During the COVID-19 pandemic a significantly high mortality rate was noted in myeloma patients. Therefore, regular covid-19 vaccination is recommended to reduce severity of COVID-19 infection.

Nervous system involvement

Spinal cord compression from a plasmacytoma, which occurs in about 10% of patients, is the most frequent and serious neurological complication in MM. The thoracic spine is the most common site of involvement, followed by the lumbar region. The clinical picture of spinal cord compression consists of back pain and paraparesis. Although it can evolve for several days or even a few weeks, the onset can be abrupt, resulting in severe paraparesis or paraplegia in a few hours. The picture is usually accompanied by a high sensory level. Lumbar involvement can cause a cauda equina syndrome (low back pain with radicular distribution and leg weakness).

Spinal cord compression is an emergency requiring immediate medical action. When suspected, urgent magnetic resonance imaging (MRI) should be performed. If confirmed, treatment with high-dose dexamethasone must be started immediately, followed by progressive tapering. Simultaneous local radiation therapy should be started as soon as possible. If the spinal cord compression is caused by a vertebral collapse or by spinal instability rather than a plasmacytoma (which is very rare), urgent surgical decompression followed by fixation of a prothesis of bone graft or methacrylate is required.

Nerve root compression can cause back pain that follows a radicular distribution with or without radicular sensory involvement. Treatment with radiation therapy is helpful, alongside use of Gabapentinoids allowing time for the action of systemic chemotherapy. Clinically significant peripheral neuropathy (PN) is very uncommon in newly diagnosed patients with MM. Leptomeningeal involvement in the central nervous system (CNS) with detection of plasma cells in the cerebrospinal fluid (CSF) is exceedingly rare. The frequency of CNS involvement is roughly 1%, the most frequent presenting features being paraparesis, symptoms from increased intracranial pressure, cranial nerves palsies and confusion. The CSF examination frequently shows plasma cells of plasmablastic morphology, as well as an increased protein concentration with positive immunofixation for the myeloma protein. Unfortunately, despite active treatment measures such as intrathecal therapy (methotrexate, cytarabine, glucocorticoids), cranial or even craniospinal irradiation, the prognosis is extremely poor with a median survival of months to a few years from the diagnosis of CNS involvement.

Prognostic factors

The outcome for patients with MM is highly heterogeneous, with survival duration ranging from a few months to more than 10 years. This heterogeneity relates to: (i) specific characteristics of the tumour itself, (ii) host factors and (iii) a series of factors resulting from the interaction between the tumour clone and the host, which mainly reflect tumour burden and disease complications (Table 28.6).

Host factors

The favourable influence of a good performance status (ECOG ≤2) and young age (<65–70 years) is well established. In contrast, neither sex nor race has prognostic influence. Immune surveillance, by T and natural killer (NK) cells, plays a relevant role in most malignancies, and low numbers of mature NK and CD4 cells have been reported in advanced-stage MM. In addition, patients who develop expanded T-cell clones (CD8+CD57+CD28−), which can recognise autologous idiotypic immunoglobulin structures, display favourable outcomes. Poor renal function due to disease or general health limits ability to give combination antimyeloma therapy and patients often have higher rates of side effects. Frailty measured by specific indices appear to be a better predictor of survival in older myeloma patients as this correlates with ability to receive therapy and toxicities from therapy.

Malignant clone factors

The second cohort of prognostic factors are those that reflect specific characteristics of myelomatous plasma cells and include morphology, immunophenotyping, cytogenetics, oncogenes, multidrug resistance and proliferative activity of plasma cells. Immature/plasmablastic morphology is associated with poor outcome. With regard to antigen expression, it has been observed that downregulation of CD117 (c-Kit) and CD56, and expression of CD28 and CD19, are associated with poor prognosis. As occurs with acute leukaemia, cytogenetic abnormalities are the most important prognostic markers for MM. Nowadays, cytogenetic evaluation is mandatory in all patients with newly diagnosed MM and should always include FISH in purified PCs or in combination with immunofluorescent detection of light-chain-restricted PCs (cIg-FISH). Amongst *IGH* translocations, patients with t(4;14) treated with either conventional or intensive chemotherapy display shorter event-free survival and overall survival. However, recent analysis supports the notion that patients with t(4;14) make up a heterogeneous group. Thus, the French group has discriminated a subgroup of these patients (approximately 45%) with both low β2-microglobulin and high haemoglobin levels at diagnosis, which displays prolonged survival after tandem transplant and

Table 28.6 Prognostic factors in MM.

Prognostic factors	Tumour related	Host related	Tumour burden
Essential	Cytogenetics/FISH: t(4;14), t(14;16), t(14;20), *17p deletions, 1q gain/ amplification, 1p deletions*	Age, performance status	International staging system (β_2-microglobulin/albumin)
Additional	Hypodiploidy LDH Plasma cell proliferation	Frailty Renal function	
New and promising	More than 1 high risk abnormality, myc aberrations	Immune status	Circulating clonotypic plasma cells

benefits from high-dose therapy. The association of t(14;16) with prognosis has not been well supported because of its low frequency. According to Mayo Clinic data, t(14;16) was linked to poor outcome in the context of conventional chemotherapy. However, controversial results have been reported using autologous transplant: the IFM group did not confirm the poor prognostic value of t(14;16) in patients receiving a tandem-autologous transplantation approach, whereas a recent study from the MRC showed a significantly shorter survival amongst patients with t(14;16) treated with autologous transplant. In contrast to t(4;14) and t(14;16), the presence of t(11;14) was shown to have either a favourable or an irrelevant influence on prognosis. Initial studies indicated that monosomy 13/del(13) was associated with poor outcome; however, analyses based on large series of MM patients have revealed that the presence of monosomy 13/del(13) as a unique abnormality, without concomitant *IGH* abnormalities, is not indicative of unfavourable prognosis. In contrast, del(17p) (with deletion of the *TP53* gene) is one of the most, if not the most, adverse prognostic factor in MM. It seems that the higher the proportion of PCs bearing 17p deletions (55% or more), the shorter the survival. Several studies have shown that 1q gains have a significant and independent poor prognostic factor; in addition, the IFM group has reported that 1p deletions are a major independent prognostic factor. A combination of ISS3 and chromosome 1q amplification has been described as a 'double hit' feature associated with poor survival. Moreover, the presence of complex, as well as non-hyperdiploid karyotypes also predict treatment failure. In contrast, hyperdiploid tumours with multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19 tend to have a favourable prognosis. Finally, the proliferative activity of the malignant plasma cells, as assessed either by the labelling index with bromodeoxyuridine or by flow cytometry, is an important prognostic markers but not routinely analysed.

One of the aims of cytogenetic classification is to define a high-risk group that should be managed differently from standard-risk patients. According to IMWG recommendation, the term 'high-risk MM' should include those patients with at least one of the following features: deletion of 17p, t(4;14) or t(14;16), detected by FISH analysis. Hypodiploidy defined by karyotyping is also used by the Mayo Clinic to define high-risk patients. Recently, a prognostic model in MM based on the frequently associated genetic lesions has been proposed. Accordingly, three genetic risk groups were defined: a favourable risk group with no adverse FISH lesions, an intermediate group with one adverse genetic lesion – t(4;14), t(14;16) or t(14;20); 17p deletion or 1q gain/amplification – and a high-risk group with more than one adverse genetic lesion.

Tumour burden and disease complications

A high proportion of plasma cells in the bone marrow, diffuse bone marrow infiltration and the presence of circulating plasma cells reflect a high tumour burden, but their prognostic influence is modest. Similarly, the impact of skeletal lesions, evaluated by radiography or bone resorption markers, is not clear. In contrast, disease complications such as anaemia, thrombocytopenia and particularly renal insufficiency have a major influence. Nevertheless, the most important factor is the concentration of β2-microglobulin, which increases as a result of both growth of tumour burden and deterioration in renal function. The higher the value, the worse the prognosis. Nevertheless, β2-microglobulin is not helpful for disease monitoring. C-reactive protein is a surrogate marker for IL-6 (a major plasma cell growth factor), which also correlates with outcome. In contrast to hypoalbuminaemia, neither the amount of paraprotein nor its isotype influences prognosis.

Only a few of the prognostic factors have real independent value. A summary of the most important is shown below:

1 Two host factors (*age* and *performance status*) reflect the ability of the patient to tolerate chemotherapy

2 Two intrinsic characteristics of the malignant clone (*cytogenetics* and *plasma cell proliferative activity*)

3 One biochemical marker that reflects tumour burden (β_2*-microglobulin*).

The International Staging System (ISS), derived from more than 11,000 patients, has shown that β2-microglobulin and albumin are the best combination of easily available markers for discriminating prognostic subgroups: stage I (β2-microglobulin <3.5 mg/L, albumin >3.5 g/dL); stage III (β2-microglobulin >5.5 mg/L); stage II (the rest). This substitutes for the Durie and Salmon classification, which affords little prognostic information. The International Staging System incorporates both ISS and LDH levels and/or genetic features. They defined the following three groups: revised ISS (R-ISS) I including ISS stage I (serum β2-microglobulin level <3.5 mg/L and serum albumin level ≥3.5 g/dL), no high-risk Cytogenetic abnormalities [del(17p) and/or t(4;14) and/or t(14;16)], and normal LDH level (less than the upper limit of normal range); R-ISS III, including ISS stage III (serum β2-microglobulin level >5.5 mg/L) and high-risk Cytogenetic abnormality or high LDH level; and R-ISS II, including all the other possible combinations. At a median follow-up of 46 months, the 5-year OS rate was 82% in the R-ISS I, 62% in the R-ISS II, and 40% in the R-ISS III groups. This is now routinely used in both clinics and myeloma trials.

Response to therapy as a prognostic factor

In addition to all the variables that can be measured at diagnosis and which have already been mentioned, response to front-line therapy represents one of the most important prognostic factors in most haematological malignancies. In the case of MM, there is still open debate about the importance of the depth of response on patient outcome. The reason for this controversy is probably that, historically, until the introduction of high-dose

chemotherapy, CR was extremely rare and the only available comparison was between responding patients (achieving partial or minor responses) and non-responding patients, with the former having a better outcome. Now patients have very high CR rates and up to a third of patients at minimal residual disease negative by flow or next-generation sequencing of VDJ clonotypes. A recently published meta-analysis, using cooperative trials for which outcome data were reported, has shown a very significant association between the minimal residual disease (MRD) negativity after treatment and the two main long-term-outcome parameters, i.e. overall survival (OS) and time to progression-related events. However, it is clear that more sensitive techniques applied regularly (e.g. multiparametric immunophenotyping [MRD flow], molecular analysis of bone marrow plasma cells by NGS, serum free light-chain test and imaging techniques such as MRI or PET to detect myeloma activity outside the bone marrow) are needed to evaluate sustained response in MM and these may contribute to a better assessment of the impact of response resulting in durable outcome such as operational cure. Both the Spanish and UK groups have shown that when transplanted and elderly MM patients show undetectable residual myelomatous plasma cells by immunophenotyping, progression-free survival (PFS) and OS are significantly longer, and this parameter is significantly more powerful than negative immunofixation.

Criteria of response

Response criteria were initially developed by the Chronic Leukaemia and Myeloma Task Force (CLMTF) in 1968. The main response parameter was a minimum 50% reduction in the M-protein. In 1972 the Southwest Oncology Group (SWOG) defined partial response (PR) as a reduction of at least 75% in the calculated serum paraprotein synthetic rate and/or a decrease of at least 90% in urinary light-chain urine protein excretion sustained for at least 2 months. The Medical Research Council (MRC) introduced the concept of plateau phase, defined as a period of stability after chemotherapy in which tumour progression does not occur, despite the presence of measurable disease. The minimum period of stability required for definition of plateau phase was 3 months. Since CRs were rarely observed with the classical conventional dose chemotherapy, neither the CLMTF nor the SWOG response criteria included a definition of CR. In addition, there were no definitions for relapse and progression.

EBMT criteria for response, relapse and progression

With the introduction of high-dose therapy/SCT, the M-protein disappears in a significant number of patients, a fact that is associated with a significant prolongation of survival. In this context, the European Group for Blood and Marrow Transplantation (EBMT) developed new criteria defining CR (negative immunofixation in serum and urine and less than 5% bone marrow plasma cells), PR (≥50% reduction in serum M-component and ≥90% in 24-hour urine Bence–Jones proteinuria) and minimal response (MR) (25–49% decrease in M-component), as well as criteria for relapse (reappearance of M-protein by immunofixation in patient who had achieved CR, and progression from PR or MR). Any type of response should be maintained for a minimum of 6 weeks. These criteria have been shown to be useful and reproducible in both transplant and non-transplant series as well as in prospective clinical trials.

IMWG response criteria

The IMWG has expanded the EBMT criteria by adding the categories of stringent CR (sCR) and very good partial response (VGPR) (Table 28.7). Patients with negative immunofixation in serum and urine and with a normal free light-chain ratio are considered in sCR. The free light-chain measurement has also been included for the evaluation of response in patients with non-secretory and oligosecretory disease. VGPR requires a decrease in the M-protein size of at least 90%. In addition, time to event, duration of response, clinical relapse and time to alternative therapy are emphasised as critical endpoints. More recently, the IMWG has revised the criteria in order to account not only biochemical assessment of response, but to now include criteria for MRD assessment (minimal residual disease) of CR using one of 2 different methods. While it is clear that PFS and OS are superior for patients who achieve MRD negativity, the depth required for long-term disease control (or cure) remains unclear. Sensitivity at a level of 10^{-5} may be useful for regulatory endpoints such as drug approvals or for comparison between two different treatment approaches, but as we seek to eliminate the residual clone and discontinue therapy, using deeper measures such as 10^{-6} or greater and sustained MRD negativity as benchmarks will be increasingly important.

Treatment

In this section, we focus on the treatment of the malignant clone, since the management of disease complications (anaemia, renal insufficiency, bone disease) has been discussed above.

Melphalan–prednisone (MP) was introduced for the treatment of MM in the late 1960s. In the subsequent 30 years, treatment improvements remained stagnant, since more complex chemotherapy combinations (e.g. VCMP, VBAD, VAD) only led to small increases in the overall RR, but without differences in survival, as assessed in a large meta-analysis that included over 6000 patients. The next step forward was the use of high-dose

Table 28.7 IMWG Standard and MRD response criteria.

IMWG standard response category*	Criteria	IMWG MRD category	Criteria – requires patient is in a CR standard CR
Stringent CR	As above, *plus* Normal free light-chain ratio Absence of clonal plasma cells†	Sustained MRD Negative	MRD negativity in the marrow (Flow or Sequencing) and by imaging confirmed at a minimum of 12 months apart
Complete remission (CR)	Negative immunofixation (serum and urine) <5% bone marrow plasma cells No soft tissue plasmacytomas	Flow MRD-negative	Absence of phenotypically aberrant clonal plasma cells by NGF on bone marrow aspirate using EuroFlow procedure (or validated equivalent method) with a minimum sensitivity of 10^{-5}
Very good partial response	≥90% decrease in serum M-protein Urine M-protein <100 mg per 24 hours	Sequencing MRD-negative	Absence of clonal plasma cells by NGS on bone marrow aspirate in which presence of a clone is defined as less than 2 identical reads obtained after DNA sequencing of bone marrow aspirates using the LymphoSIGHT platform (or validated equivalent method) with a minimum sensitivity of 10^{-5}
Partial response	≥50% decrease in serum M-protein ≥90% decrease in urine M-protein or <200 mg per 24 hours ≥50% decrease in soft tissue plasmacytomas	Imaging plus MRD-negative	MRD negative as defined by NGF or NGS plus disappearance of every area of increased tracer uptake found at baseline or preceding PET/CT or decrease to less than mediastinal blood pool SUV or decrease to less than that of surrounding normal tissue.
Minimal response	≥25% but ≤49% reduction in serum M protein and reduction in 24-hour urine protein by 50–89% If present at baseline, ≥50% reduction in size of soft tissue plasmacytoma		
Stable disease	Not meeting the above response criteria or progression criteria		

* All response categories require two consecutive measurements made at any time.
† Determined by immunohistochemistry or immunofluorescence. (*Source:* Adapted from Kumar *et al.* Lancet Oncology March 2016.)

melphalan followed by stem cell support (autologous SCT) for fit myeloma patients, which resulted in a significant improvement in disease-free survival and OS. However, for elderly patients, MP remained the standard of care until it was replaced by the use of lenalidomide/dexamethasone in several randomised trials. Now the major questions in the field revolve around the use of combinations with len/dex for frail patients, and the use of three or four drug combinations as part of induction and risk-adapted maintenance for fitter patients with newly diagnosed myeloma.

In this section, we first discuss the treatment of newly diagnosed patients stratified according to fitness status with age as a variable but not the sole determinant of treatment decisions, and then analyse the options for relapse/refractory patients, as well as emerging novel agents.

Should all myeloma patients be treated?

Currently, only myeloma patients with symptomatic disease (defined by CRAB criteria) should be treated, patients with

smouldering myeloma are not treated until they develop symptomatic disease. Attempts at early intervention in SMM patients with alkylating agents, bisphosphonates and antagonists of the receptor of interleukin 1β or thalidomide failed to show a significant benefit. However, none of these studies discriminated between high-, standard- or low-risk SMM patients, and all used agents that carried modest single-agent activity, and toxicity. By contrast, the Spanish group conducted a Phase III randomised trial focusing only on high-risk smouldering myeloma patients, comparing early treatment with lenalidomide–dexamethasone induction therapy followed by lenalidomide maintenance therapy versus observation for a 2-year defined period of treatment. The results showed that the experimental arm was associated with a significant delay in progression to symptomatic myeloma (3 years after study entry, 77% of patients in Group A and 30% in Group B were progression-free; hazard ratio, 5.59; $P < 0.001$). This delay translated into a significant overall survival benefit (the proportions of patients alive at 3 years was 94 and 80%, respectively; hazard ratio, 3.24; $P = 0.03$). Newer updates of this data suggest that the difference in PFS and OS remains at 7 and 10 years follow-up, and more importantly, the use of lenalidomide did not shorten second remission duration (PFS2) refuting a concern that patients would develop drug resistance and then be less responsive to additional therapy. More recently the ECOG group in the US published results from another phase 3 randomised trial also evaluating patients with intermediate and high-risk SMM who were randomised to receive either lenalidomide alone or observation. In this trial, it was also demonstrated that there was a significant delay in time to developing MM with a similar hazard ratio to the Spanish trial. OS analysis was too soon to evaluate but was not worse for patients who received lenalidomide alone. On the heels of these 2 trials, there are now several trials ongoing evaluating new antibodies targeting CD38 with lenalidomide, as well as more intensive 'myeloma like' treatments that are currently ongoing and are seeking to challenge the notion that patients with high-risk SMM should only be observed. In the context of these small phase 2 studies, as well as now 2 large phase 3 trials demonstrating benefit for early intervention, it is reasonable to discuss with patients about the pros and cons of limited duration therapy with either lenalidomide or len/dex as an attempt to delay progression to MM. Whether more intensive approaches are more successful than the prevention approaches described above will need to wait for longer follow-up and phase 3 trials before they become more widely used.

Treatment of newly diagnosed transplant candidate patients

Currently, treatment in this setting usually includes four to six cycles of induction therapy, followed by autologous SCT and maintenance.

Induction therapy

The history of induction therapy and myeloma was focused on changing the recipe of akylators and steroids used in combinations to debulk the disease prior to stem cell collection and transplant. This included combinations such as VAD, VBMCP and others that used cyclophosphamide as part of the initial induction therapy. With the observation that thalidomide had activity in refractory MM, it too was used with steroids and alkylators, but these regimens were limited by toxicity and lacked the real efficacy that we have now come to expect from induction regimens using modern agents. The current standard of care for a fit MM patient undergoing induction therapy is at a minimum a combination of an immunomodulatory agent such as lenalidomide (or thalidomide) with a proteasome inhibitor such as bortezomib (or carfilzomib). The regimen with the largest data set supporting its use in phase 2 and 3 trials is the RVD regimen combining bortezomib and lenalidomide with dexamethasone, and in the initial phase 2 study had an overall response rate of 100% with a very high VGPR or better rate. The advantage of RVD over its European counterpart of VTD (substituting the lenalidomide with thalidomide) is less neurotoxicity and higher efficacy as manifested by deeper responses. Based on data from the SWOG 0777 trial and others, RVD was demonstrated to be superior to the doublet of len/dex in terms of both PFS and OS. Since the establishment of a non-chemotherapy-based triplet as a standard induction regimen, variations on the agents used have also occurred and led to the creation of the KRD regimen, a combination that substitutes the bortezomib with carfilzomib. Several phase 2 studies demonstrated better depth and overall responses with less neuropathy when KRD was used compared to historical data using RVD. The ECOG cooperative group led a randomised phase 3 trial for standard-risk patients and compared RVD with KRD to address this question and demonstrated equivalence with more neuropathy in the RVD group, and more cardiac and pulmonary toxicity in the KRD group. While the ORR and VGPR rates were higher with KRD, this did not translate into an improvement in PFS in the trial. The differences between three-drug regimens using one or the other proteasome inhibitor have now given way to a larger discussion regarding the incorporation of monoclonal antibodies targeting CD38 into the mix as part of a four-drug induction for fit myeloma patients with planned transplant. The largest of these trials, the Cassieopia trial, compared VTD +/− Daratumumab as induction followed by transplant, consolidation with VTD +/− Daratumumab and then a second randomisation between data and observation. The second randomised has clouded the ability to interpret PFS as an endpoint for this study but clearly one can see differences in response rates and depth of response favouring the use of adding Dara to the induction regimen of VTD prior to transplant. In a more US styled trial, patients were randomised to

either RVD+/− dara induction, transplant, RVD+/− dara consolidation, and then either len+/− dara maintenance. This trial confirmed the improvement in depth and overall response rate with significantly higher MRD negative rates amongst the patients in the dara arm, and after 2 years of follow-up there is now a suggestion that the PFS is beginning to separate. These trials have now established that quadruplet-based inductions will likely become adopted more broadly, at least in the induction phase of treatment.

Autologous stem cell transplantation

High-dose therapy followed by autologous SCT was demonstrated to improve OS compared with standard-dose therapy in prospective randomised trials conducted by the French (IFM) and English (MRC) groups, and has revolutionised the care of patients with myeloma. The use of age as a determining factor for eligibility is one that has predominated in Europe but has in part given way to one that is more focused on using performance status as a guideline rather than age alone. In the US, patients are considered eligible for SCT up to 79 years of age, presuming adequate organ function and performance status, though the dose of melphalan may be reduced for older patients or for patients with significant comorbidities (Mel 140 mg/m^2 versus Mel 200 mg/m^2) such a chronic renal failure.

The question at hand currently is about the benefit of HDT in the era of modern induction. The continued use of HDT initially is based on inducing a higher rate of CR, something historical induction regimens were not able to do with any significance. However, in an era where we are using IMID/PI/Dex combinations are frontline therapy and have reduced or eliminated the routine use of alkylator-based inductions, the speed and depth of response is much higher than we have seen before, and again the benefit of HDT questions have arisen. The first trial to really address this was the IFM 2009 trial, a randomised trial of RVD induction followed by either transplant, or continued RVD induction and then maintenance therapy. In each of the reported updates so far, there remains a benefit for the group that underwent early HDT in terms of PFS compared to the patients who were treated with RVD alone. In the most recent update, even amongst patients who achieved MRD negativity, those who underwent HDT had a longer PFS than those who did not. While there is no difference in overall survival between the 2 arms, given that the median OS for MM patients is now well beyond 10 years, the use of OS as a metric of success in this patient population is perhaps unrealistic, and improving PFS is the better endpoint. In the RVD 1000 series from the group at Emory, median PFS for all patients is 65 months with continuous maintenance, demonstrating that prolonging PFS in this group really should be a major goal, presuming OS for the transplanted group is not worse, which has not been demonstrated in modern series.

A second trial asked a similar question but with a different induction regimen, In the FORTE trial, the use of KRD was tested in 2 arms, with a 3rd arm using KCD as induction. In one of the KRD arms, patients were transplanted, in the other, they were not. In the KCD arm, all patients underwent transplant. While the overall response rate for the KRD arms were similar (with or without transplant) the group that did not have a transplant again had a shorter PFS and sustained MRD negativity again supporting the use of HDT even with highly active induction regimens. The KCD arm was inferior in every analysis, though in some settings it was similar to the KRD with no transplant arm. While these trials do not reflect all the new and different induction regimens currently available and do not address the addition of daratumumab or other CD38 targeting antibodies, to date there is no randomised trial suggesting that transplant does NOT offer benefit even in the context of highly effective induction therapy, and thus, should be considered for all patients seeking to prolong their first remission and maximise the initial depth of response to treatment.

With regard to tandem autologous SCT, there has been a resurgence, particularly in Europe, for its use in high-risk MM. This will be discussed in greater detail below, however at this time, unless patients have not responded to standard novel agent-based treatments, or do not have access to triplet maintenance in the context of high-risk disease, the routine use of tandem autologous transplant is to be discouraged.

Consolidation and maintenance

Consolidation and maintenance are areas of treatment that have rapidly advanced in the past decade based on small phase 2 studies as well as larger phase 3 experiences. In the context of consolidation, we are classically referring to treatment given after 1 cycle of HDT and transplant, but before maintenance starts. A large BMT CTN trial compared the use of RVD consolidation vs a second autologous transplant (tandem) versus no consolidation and proceeding directly to maintenance therapy. The maintenance in each arm was the same, lenalidomide, and following the first 2 arms of treatment, patients also received lenalidomide maintenance. In this trial, there was no difference in progression free survival or overall survival suggesting that there was no real benefit for consolidation, and this has become the standard approach in the US.

At the same time, the EMN was also doing a trial of tandem autologous transplant vs single transplant as consolidation, with all patients receiving some form three drug consolidation after transplant. In their analysis, there was a benefit for tandem autologous transplant, most noted in the high-risk patient population, however this must be put into context. In the EMN trial, most patients received VCD induction, which we know from randomised trials is inferior to VTD or VRD, and thus they received suboptimal induction. In the BMTCTN study, most patients received VRD induction, and many received more than four cycles of induction suggesting they were in a lower disease

burden state prior to undergoing HDT and transplant. Given very strong OS benefit associated with lenalidomide maintenance, and emerging data supporting the use of PI/IMID +/− dex as maintenance for high-risk patients, the absolute benefit of consolidation with a second transplant remains unclear. At this time, if a patient is not able to receive three drug maintenance with high-risk disease, a second autologous transplant could be considered, but if they are able to receive either VRD or KRD as consolidation post-transplant, this too may be sufficient to reduce the risk of relapse in a high-risk patient population.

Allogeneic stem cell transplantation

Allogeneic SCT, in the context of so many novel agents have clearly fallen to the side as a routine approach for patients with myeloma. Even amongst younger patients with high-risk disease, their relative benefit from an allogeneic transplant is limited by a remaining high risk of relapse, GvHD, and death as a treatment related event when compared with standard and new treatment approaches. The benefit of allogeneic transplant appears to be minimal in the setting of highly proliferative myeloma, often the worst amongst the high- risk groups, and in the era of novel bispecific, CAR T-cells, and other new precision and immune based treatments that are currently in the armamentarium for myeloma patients, the indications for the routine use of allogeneic transplant are non existent outside the context of a well-designed and hypothesis driven clinical trial.

Treatment of newly diagnosed elderly and non-transplant candidate patients

Melphalan + prednisone (MP) has been the gold standard for over 40 years; however, the scenario has completely changed with the introduction of novel agents such as thalidomide or bortezomib, lenalidomide and now daratumumab.

While many trials over the past decade added in novel agents with the MP backbone (MP + bortezomib, MP + thalidomide, MP + lenalidomide, MP + carfilzomib), each demonstrating superiority over the use of MP alone, there was a movement to eliminate the use of MP in total from regimens for older/frailer patients. This was initiated in the E4A03 which took all newly diagnosed MM patients regardless of performance status, and randomised them to receive lenalidomide with either high dose or low dose dexamethasone. This trial alone ushered in a new era of non-aklylator based treatments for older patients. Subsequent to this trial, the FIRST trial was completed that compared 3 arms:MPT, RD for 18 months(RD18) 18 or RD continuous therapy. This demonstrated that both RD arms was superior to MPT in terms of PFS, and that PFS appeared to be longer with the use of continuous therapy with RD. These 2 trials combined with the SWOG 0777 trial that also evaluated both older and younger patients with either RVD or RD without transplant further cemented in the notion that MP based regimens are no longer needed in order to gain long remissions, and by avoiding the use of alkylators, patients are able to enjoy even longer remissions and stay on continuous therapy. In an effort to incorporate the CD38 targeting antibody daratumumab into treatment for older/frail patients, the ALCYONE trial was initiated that randomised patients to receive either MPV or MPV+ daratumumab. In this trial, the depth and speed of response was significantly higher with the addition of daratumumab, and this translated into a higher MRD negative rate, higher CR rate, and a longer PFS. Additionally, with longer follow-up, there is now an overall survival benefit for the use of D MPV vs MPV alone, however this regimen remains a throwback to the era of using MP based backbones, and in the US gets very little use. Most recently, RD was compared in the MAIA trial with Daratumumab + RD (DRD), and in the initial paper as well as subsequent updates, a very long PFS is being reported, more than 60 months, suggesting that not only is the use of a monoclonal antibody such as daratumumab highly effective in deepening the response, but that the safety profile when combined with RD is maintained, allowing even older patients to tolerate this treatment and stay on therapy for 5 years.

A major question that is being asked at this point is if we are ready to omit the use of proteasome inhibitors as initial therapy for older frailer patients given the very long PFS and depth of response seen with DRD. While DRD has not been compared head to head with VRD, the PFS from the SWOG trial suggests that VRD with len maintenance is around 36 months, while the MAIA trial suggests with both dara and len, the PFS is closer to 60 months. However, it is important to note that in the MAIA trial, the proportion of genetic high-risk patients was very low, and thus the efficacy for either arm in the high-risk group cannot be easily quantified. Thus, one approach may be to reserve the use of initial proteasome inhibitor for patients who have high risk genetics at presentation, while others may be safely treated with DRD. Given the large amount of data and safety on VRD for older patients, this may be the treatment of choice for the high-risk group, though there remains no data for high risk older patients in a randomised setting to confirm this approach. Alternatively, in a randomised trial of MVP vs carfilzomib + MP, it appears that MPV was superior from a safety aspect suggesting that perhaps carfilzomib in current doses and schedules or combinations is not an optimal approach for induction of the older frail patient.

Treatment at relapse

The landscape for management of relapsed myeloma varies by a number of different factors that all go into play when defining next treatment steps. There has been an explosion of several large randomised phase 2 and 3 trials in this space, that complicates choice of treatment, and thus paying specific attention to three types of factors: patient-related factors (renal function, hepatic function, comorbidity and patient preference); disease related factors (nature of relapse, genetic risk/stage, disease

burden), and treatment related factors (previous drug therapy and resistance, treatment related toxicity, duration and depth of previous response). Each of these are weighed in the context of relapse and balanced with the weight of available evidence that may support how to best move forward for any given patient.

In the setting of current myeloma therapy, where an immunomodulatory agent is combined with a proteasome inhibitor for induction therapy, there are three options for the 'backbone' of salvage therapy. These can include a proteasome inhibitor (bortezomib, ixazomib, or carfilzomib), an immunomodulatory agent (lenalidomide or pomalidomide) or a CD38 directed monoclonal antibody (daratumumab or isatuximab). Amongst these 3, if a patient is not resistant to an anti-CD38 Moab, then this becomes the backbone and either an IMID or PI is added to it, and each of these scenarios has phase 3 randomised data supporting its use. If a patient is sensitive to lenalidomide, the longest PFS in early relapse is the combination of daratumumab with len/dex, but since most patients who are relapsing are progressing on continuous len maintenance, the options that are often considered are carfilzomib/CD38/dex or pomalidomide/CD38/dex. There are other non-CD38 based triplet combinations that have demonstrated benefit in randomised phase 3 trials over doublet regimens but given the very high activity and PFS favouring CD38-directed salvage therapy, it would seem that amongst 3 highly active classes, using a CD38 as the central part of a regimen and partnering with either an IMID or PI makes the most sense.

In the context of progressive relapse, again going to the three factors that help define how to approach a patient is the standard, but in this setting, the list of agents that a patient with myeloma is resistant to is a much larger list, and so using remaining sensitive agents in combinations makes the most sense. If a patient received IMID/PI induction, then at salvage received PI/CD38, that leaves an IMID backbone for salvage 2 with other available agents, or if IMID/CD38 was used in salvage, then PI backbone becomes the next step. Once a patient develops myeloma that is triple class refractory (resistant to PI, IMID and CD38 antibody, the choices of options are much more limited. In the US options at this point that are approved are 2 BCMA directed approaches, an antibody drug conjugate (ADC) and a chimeric antigen receptor T-Cell (CART), and the other is a new agent Selinexor, which targets expo1. All three of these agents approved in refractory myeloma were approved in the context of five or more prior lines of treatment and have different pro's and con's for their use.

Belantamab mafadotin is a BCMA directed ADC that has activity in refractory myeloma, and while it does have deep and durable responses in many patients, does have a new side effect that impacts visual acuity through corneal microcysts. Fortunately, this adverse event is reversible and can be managed in partnership with an ophthalmologist along withdose holds and dose modifications.

Selinexor is an oral agent that also has activity when combined with dexamethasone in the context of refractory disease, and also has significant activity when combined both with proteasome inhibitors (bortezomib and carfilzomib) as well as with pomalidomide. The advantage of the combinations is that it allows one to use once a week dosing, which seems to improve the adverse event profile and is synergistic with agents in myeloma. Adverse events with Selinexor include nausea, diarrhoea, anorexia, weight loss and fatigue. These can be mitigated with aggressive supportive care measures that are to be used preemptively for the management of patients. With lower doses, combination therapy and aggressive supportive care many patients can gain the benefit of this new target and treatment even with refractory myeloma.

Idecabtagene vicleucel (ide-cel) is a CART directed at BCMA and is now FDA-approved for the management of patients with triple class refractory myeloma. The efficacy of ide-cel is quite impressive with a very high overall response rate, and the added advantage of not requiring additional therapy once the cells are delivered as part of a 'one and done' treatment approach. While there is lymphodepletion therapy (LD) this typically uses low doses of cyclophosphamide and fludarabine that can be administered as an outpatient. The most common adverse events related to CART therapy are cytopenias, cytokine release syndrome (CRS) and neurotoxicity (ICANS). There are specific grading criteria for CRS and ICANS, but overall, the severity of both of those are lower than is seen with CARTs used targeting CD19 in ALL or NHL.

Side-effects associated with novel agents

Because of the previous history of thalidomide, a major concern was its toxicity profile. The side-effects are dose related and the most common include constipation, weakness, somnolence and neuropathy. Peripheral neuropathy is a common adverse event with thalidomide therapy and often limits the dose and duration of treatment. The use of combination therapy has raised concerns about an increased risk of DVT. Apparently, the major risk of DVT occurs when tumour load is high and thalidomide is combined with chemotherapy, especially adriamycin. Accordingly, in this setting, anticoagulant prophylaxis with low-molecular-weight heparin (LMWH) or aspirin is mandatory. Current data suggest that lenalidomide is better tolerated than thalidomide in several aspects: it does not usually produce clinically significant somnolence, constipation or neuropathy, although the incidence of myelosuppression is higher, mainly neutropenia (grade 3 in 17–30%) and thrombocytopenia, which are manageable with dose reduction and growth factor support. Similarly to thalidomide, lenalidomide is associated with a higher risk of DVT (5–25%) and the risk increases in patients with comorbidities, previous history of DVT, concomitant use of erythropoietin, high-dose dexamethasone, anthracyclines or high tumour mass. For these reasons, anticoagulant prophylaxis with LMWH or aspirin is mandatory. The most frequent toxicities of bortezomib include fatigue, gastrointestinal symptoms, cyclical thrombocytopenia

and, particularly, peripheral neuropathy. This latter side-effect, classified as grade 3 in 9–20% of patients, is the main reason for treatment discontinuation, and the early detection of peripheral neuropathy is most important in order to reduce the dose or frequency of injections. The use of a weekly schedule of bortezomib and particularly the subcutaneous administration has resulted in a significant decrease in peripheral neuropathy. Concerning panobinostat, thrombocytopenic and gastrointestinal effects are the most relevant toxicities. Elotuzumab and daratumumab can produce infusion reaction.

Promising new drugs

The field of new drugs is one that is rapidly expanding in the past few years on top of an already rapidly growing list of treatment options for patients. Most notable has been the explosion of immune-based treatments that first started with the unconjugated monoclonal antibodies targeting CD38 and SLAMF7, but has more recently grown to include agents that target BCMA, GPRC5D and FCRH5. Additionally, there are several signalling or newer generation versions of older agents that also have shown promise (non-immune) and are worth discussing in this section as well

Immune-based treatments

Chimeric antigen receptor T-Cells

In the area of BCMA-directed CART, there are a number of autologous and allogeneic CART products that are currently being developed. The field was rapidly advanced with the development and approval of CD19-directed CART in ALL and NHL, and with that initial experience, BCMA was the first target used in the context of myeloma. In the autologous setting, the advantages include lack of GvHD or rejection, which results in a longer half-life for the product when compared with allogeneic sources for CART, which have the advantage of a higher quality T-cells (fewer exhaustion phenotype T-cells) and more rapid availabilty (off the shelf). In the context of refractory disease, overall response rate is typically what is used to assess efficacy, but in the area of CART therapy, the overall response rate is usually >70% for most products, making that assessment less useful for identifying differences between products. The Karmma trial enrolled a group of refractory myeloma patients to receive one of three different CART doses following standard lymphodepletion therapy. The overall response rate for all doses and patients was 70% with a median duration of response (DOR) of 11 months. In the Cartitude 1 trial using cilta cel, the overall response rate was 97% and a high fraction of those patients became MRD negative over the next few months. The median PFS is expected to be >24 months with longer follow-up. There are several other CART products in development all with high overall response rates and variable durations of response and remission. How these will compare with cilta-cel and ide-cel remains unknown as does the response to CART in earlier lines of therapy. In terms of toxicity, the most common adverse events include haematologic toxicity, cytokine release syndrome (CRS) as well as neurologic toxicity. In general, the severity of grade 3/4 CRS and neurologic toxicity is lower for BCMA-directed CART than we see with CD19-directed CART, and most patients can be managed with steroids and tociluzumab. The time to develop CRS does vary by product used with ide-cel being relatively early after infusion, while with cilta-cel occurring around 7 days after infusion. The other major adverse event that is noted is a higher incidence of infections following CART so aggressive prophylaxis for the period of time when the plasma cell suppression is most intense is recommended for all patients

Bispecific antibodies

The use of bispecific antibodies or T-cell engagers (TCE) is a treatment approach that has had significant activity in the area of ALL or DLBCL. In myeloma, there were concerns early on that in the setting of refractory MM that T-cell function may be an issue, yet in early phase 1 studies using TCEs in MM, it was identified that response rates that range from 55 to 80% even in the context of refractory MM. There are now 6 different TCEs that are directed against BCMA that are in phase 1 and phase 2 studies, and the responses and depth of response are all very encouraging with some patients achieving MRD negativity. In addition to TCEs targeting BCMA, there are two additional immune targets in development, GPRC5D and FCRH5. These targets are expressed on nearly all plasma cells, and early data with talquetamab (GPRC5D) and cevostamab (FCRH5) suggest high response rates and similar adverse events with BCMA-directed TCEs. One new toxicity noted with targeting GPRC5D is dysgusia and skin/nail issues, both of which are reported in phase 1 studies as mostly occurring as grade 1/2.

Other targets in development

Venetoclax

Venetoclax is a bcl-2 inhibitor that is approved for patients with B-cell malignancies such as CLL and indolent NHL. Unlike most lymphomas, most myeloma cells are mcl-1 dependent, limiting the activity of venetoclax for all myeloma patients. However, preclinically there are subsets of myeloma patients who are bcl-2 dependent and thus are sensitive to this treatment approach. In early phase1 development, it was clear that patients whose myeloma carried the t(11;14) translocation had a much higher overall response rate than those who did not (40 versus 5%), and this improved with the addition of dexamethasone. These early phase trials held signficant promise for Ven in a subset of myeloma patients and led to larger combination trials with daratumumab, bortezomib and carfilzomib. These early combination trials demonstrated significant activity and did so in both sensitive and resistant patients. The randomised phase 3 Bellini trial tested the hypothesis that bortezomib and dex were a synergistic partner for Ven and compared borteomib/Ven/Dex with

borteomib/dex in an unselected early relapse population. While the early phase studies suggested benefit for Ven even amongst non-t(11;14) patients, in hind site much of that activity may have been related to the partner agent (bortezomib or carfilzomib) as results from the phase 3 trial demonstrated a big difference in PFS favouring the addition of Ven, but a survival decrement for Ven treated patients. On further analysis, Bellini demonstrated significant benefit in PFS and OS for t(11;14) patients, but the survival decrement was noted in the non-t(11;14) population, which was enriched for high-risk patients. Ongoing confirmatory trials continue in the biomarker-specified population, and hopefully will result in the first precision medicine approach being approved in myeloma using venetoclax.

CelMods

Celmods represent the next generation of immunomodulatory agents (ImiDs) that come in the line of thalidomide, lenalidomide and pomalidomide. The two agents with the most clinical data in myeloma are CC-220 (iberdomide) and CC-92480. Both agents have completed phase 2 evaluation alone and with dexamethasone and have impressive overall response rates of 30% or greater in triple-class refractory myeloma. Iberdomide is currently being evaluated in combination with bortezomib, carfilzomib and daratumumab, and currently, it appears that they can be combined with full doses of both agents in refractory myeloma. Both agents are able to overcome pomalidomide resistance in some patients, and there are no new adverse events noted from these early phase trials different from other agents in this class, and neither agent has neuropathy as a significant side effect. Future trials setting up regulatory approval are in the planning for both, and not only are the exciting from the aspect of new agents that can overcome ImiD resistance but also from the immune stimulating aspect, where it appears that the ability to partner with immune targeted agents is even better than previous agents in this class setting up important combination trials in the future.

Acknowledgements

The authors would like to thank Sherin Verghese for proof reading.

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CHAPTER 29

Amyloidosis

29

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Introduction

Amyloidosis is a disorder of protein folding in which normally soluble proteins are deposited in the extracellular space as insoluble fibrils that progressively disrupt tissue structure and function. Over 30 unrelated proteins can form amyloid *in vivo*, the clinical amyloidosis syndromes are classified according to the fibril protein (Table 29.1).

Amyloid deposition is remarkable in its diversity; it can be systemic or localised, acquired or hereditary, life-threatening or merely incidental. Clinical consequences occur when accumulation of amyloid fibrils is sufficiently substantial to interfere with normal function. However, it is apparent that the disease caused by amyloidosis is not just because of the physical deposition of the protein fibrils but also due to direct proteotoxicity of the pre-fibrillary oligomers. The pattern of organ involvement varies within and between fibril types and clinical phenotypes overlap greatly. In systemic amyloidosis where virtually, any tissue may be involved, the fibrils target certain organs with greater frequency (heart, kidneys, nerves and liver). Untreated, the disease is often fatal, although prognosis has improved with increasingly effective treatments of many underlying conditions. Greater understanding of the pathogenesis of the disease, allowing improved subtyping and appropriate targeted therapies, with improved supportive care, including haemodialysis and solid organ transplantation in selected patients have also influenced the prognosis of this disorder. In localised amyloidosis, deposits are confined to a particular organ or tissue, but may have serious consequences such as haemorrhage. Localised amyloid deposition is also a pathological hallmark of uncertain significance in some important common diseases including Alzheimer's disease and type 2 diabetes mellitus.

This chapter describes the clinical features, differential diagnosis and management of systemic AL (monoclonal immunoglobulin light chain) amyloidosis, which is the most common and serious form of the disease.

Pathogenesis of amyloid

Proteins that can form amyloid can exist in two completely different three-dimensional conformations, the transformation involving massive refolding of the normal form into one that can autoaggregate in a highly ordered manner to produce the characteristic predominantly β-sheet, rigid, non-branching fibrils of 10–15 nm in diameter and indeterminate length. Acquired biophysical properties common to all amyloid fibrils include insolubility in physiological solutions, relative resistance to proteolysis, and ability to bind Congo red dye in an ordered manner that gives the diagnostic green birefringence under cross-polarised light.

Amyloid deposition can occur in three circumstances:

1 When there is a sustained abnormally high concentration of certain normal proteins, such as serum amyloid A protein (SAA) in chronic inflammation or β_2-microglobulin in renal failure, which underlie susceptibility to AA and β_2-microglobulin amyloidosis, respectively.

2 When there is a normal concentration of a normal, but inherently amyloidogenic, protein over a prolonged period, such as transthyretin (TTR) in wild-type transthyretin (previously also known as senile systemic) amyloidosis.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).

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Table 29.1 Classification of amyloidosis (by clinical frequency).*

Type	Fibril precursor protein	Clinical syndrome	Organs involved
ATTR	Normal plasma transthyretin	Senile systemic amyloidosis with predominant cardiac involvement	Heart, Carpal tunnel and lower spine
	Genetic variants of transthyretin (e.g. ATTR Met30, Ala60, Ile122)	Familial amyloid polyneuropathy (FAP), with systemic amyloidosis and often prominent amyloid cardiomyopathy	Nerves and heart
AL	Monoclonal immunoglobulin light chains	Systemic amyloidosis associated with myeloma, monoclonal gammopathy, occult B-cell dyscrasia. Formerly known as primary amyloidosis	Kidneys, heart, nerves, liver and soft tissues
ALECT2	Leucocyte chemotactic factor II	Systemic amyloidosis with predominant renal involvement	Kidney and liver
AA	Serum amyloid A protein	Systemic amyloidosis associated with acquired or hereditary chronic inflammatory diseases. Formerly known as secondary or reactive amyloidosis	Kidney (may involve other organs in very advanced stages)
$A\beta_2M$	β_2-Microglobulin	Dialysis-related amyloidosis (DRA) associated with renal failure and long-term dialysis. Predominantly musculoskeletal symptoms	Joints
AFib	Genetic variants of fibrinogen α chain (e.g. Afib Val526)	Autosomal dominant systemic amyloidosis. Non-neuropathic usually with prominent nephropathy	Kidneys
AapoAI	Genetic variants of apolipoprotein A-I (e.g. AapoAI Arg26 and Arg60)	Autosomal dominant systemic amyloidosis. Predominantly non-neuropathic with prominent visceral involvement, especially nephropathy. Minor wild-type ApoAI amyloid deposits may occur in the aorta	Kidneys, liver and heart (nerves in some mutations)
ALys	Genetic variants of lysozyme (e.g. ALys His67)	Autosomal dominant systemic amyloidosis. Non-neuropathic with prominent renal and gastrointestinal involvement	Gut, skin, liver and kidneys
AApoAII	Genetic variants of apolipoprotein A-II	Autosomal dominant systemic amyloidosis with predominant renal involvement	Kidneys
AGel	Genetic variants of gelsolin (e.g. Asn187)	Autosomal dominant systemic amyloidosis. Predominant cranial nerve involvement with lattice corneal dystrophy	Cranial nerves, cornea and skin
ACys	Genetic variant of cystatin C (Gln68)	Hereditary cerebral haemorrhage with cerebral and systemic amyloidosis	Cerebral blood vessels
AIAPP	Islet amyloid polypeptide	Amyloid in islets of Langerhans in type 2 diabetes mellitus and insulinoma	Pathogenic impact unclear

* Amyloid composed of peptide hormones, prion protein and unknown proteins not included.

3 When there is production of an acquired or inherited variant protein with an abnormal amyloidogenic structure, such as monoclonal immunoglobulin light chains in AL amyloidosis or some hereditary variants of TTR, lysozyme, apolipoprotein A-I and fibrinogen Aα chain.

Genetic and environmental factors that may influence susceptibility and timing of as well as the tissue target of amyloid deposition are unclear, but once underway, amyloid deposition is unremitting and exponentially progressive in face of ongoing supply of the precursor protein.

Amyloid deposits not only consist mainly of amyloid fibrils, but also contain some common minor constituents, including certain glycosaminoglycans (GAGs) and the normal circulating plasma protein serum amyloid P component (SAP), as well as various other trace proteins. SAP binds in a specific calcium-dependent manner to all amyloid fibrils, but not their precursor

proteins, which is the basis for the use of radiolabelled SAP for scintigraphy to image and monitor amyloid deposits. Amyloid fibril-associated GAGs mainly comprise heparan and dermatan sulfates; their universal presence, restricted heterogeneity and intimate relationship with the fibrils suggest a contribution to the development or stability of amyloid deposits.

Many of the pathological effects of amyloid can reasonably be attributed to its substantial physical presence or precise location, for example within glomeruli or nerves. The relationship between amyloid load and associated organ dysfunction differs greatly between locations and individuals, and the rate of new amyloid deposition may be an important factor. Aggregated amyloid proteins may also be directly cytotoxic, but they appear to evoke little or no local reaction in the tissues. However, clearance of amyloid deposits from tissue sites is mediated by complement-dependent macrophage activation and giant cell formation – a potential for therapeutic exploitation to accelerate amyloid clearance.

Treatments that substantially reduce the supply of amyloidogenic precursor protein frequently result in stabilisation or regression of existing amyloid deposits and are often associated with preservation or improvement in the function of amyloidotic organs.

Systemic AL amyloidosis

Systemic AL ('primary') amyloidosis is an uncommon complication of monoclonal gammopathy or multiple myeloma. With an incidence of approximately 8–10 cases per million population, it is about a fifth as common as myeloma. As a rule of thumb, for every five myeloma patients, there is one amyloid patient (often undiagnosed). AL fibrils are derived from monoclonal immunoglobulin light chains, which are unique in each patient. The uniqueness of an individual's light chain may explain the considerable heterogeneity in organ involvement and outcome in AL amyloidosis. Virtually any organ other than the brain may be directly affected, most commonly the kidneys, heart, liver and peripheral nerves. Early symptoms are non-specific, and lack of a single diagnostic test is seriously limiting for easy early diagnosis. Routine screening techniques often fail to detect the subtle underlying monoclonal gammopathy, causing delay in diagnosis and detrimental disease progression. Awareness of AL amyloidosis is thus essential, with early recourse to more informative investigations such as serum-free light chain (SFLC) analysis and target organ or screening tissue biopsies.

AL fibrils and monoclonal light chains

AL amyloid fibrils are derived from monoclonal immunoglobulin light chains and consist of the whole or part of the variable (V_L) domain with molecular mass between about 8 and 30 kDa. Monoclonal light chains are unique in structure in each individual and in their inherent propensity to form amyloid fibrils. Overall, about 1–2% of patients with monoclonal gammopathies eventually develop AL amyloidosis, but this is not possible to predict. The inherent 'amyloidogenicity' of certain monoclonal light chains has been demonstrated in an *in vivo* model in which purified Bence–Jones proteins were injected into mice. Light chains from patients with AL amyloidosis formed amyloid, whereas those from myeloma patients did not. AL fibrils are more commonly derived from λ than κ light chains, despite κ isotypes predominating amongst both normal immunoglobulins and monoclonal gammopathies. Some amyloidogenic light chains have distinctive amino acid replacements or insertions compared with non-amyloid monoclonal light chains, including replacement of hydrophilic framework residues by hydrophobic ones, changes that can promote aggregation and insolubility. Certain light-chain isotypes, notably $V_{\lambda VI}$, are especially amyloidogenic, and there is a degree of concordance between some isotypes and their tropism for being deposited as amyloid in particular organ systems. For example, the $V_{\lambda VI}$ isotype often presents with dominant renal involvement, whereas the $V_{\lambda II}$ isotype frequently involves the heart.

The plasma cell dyscrasia

Any B-cell dyscrasia, including multiple myeloma, Waldenström macroglobulinaemia and other malignant lymphomas/leukaemias, producing a monoclonal immunoglobulin may be complicated by AL amyloid deposition. Ninety percent of patients with AL amyloidosis have clonal plasma cells in the bone marrow (median plasma cell percentage ~9%) and 6% have an IgM-producing monoclonal B cell clone (typically a lymphoplasmacytic lymphoma/ Waldenström's macroglobulinaemia). Amyloid deposits can be found histologically in up to 15–20% of patients with myeloma and are generally asymptomatic but with marked improvement in the longevity of patients with myeloma, there is a potential that amyloidosis may become symptomatic later in the disease course. Molecular transcriptomic profiling reveals interesting differences between plasma cells in AL vs. those in other MGUS or myeloma (such as fourfold upregulation of glycosylation genes and downregulation of genes encoding for oxidative phosphorylation, MYC or ribosome biogenesis). Translocation t(11;14) is disproportionally common in AL (seen in ~50% of all cases). The presence of t(11;14) or hyperdiploidy is associated with a poorer prognosis in AL (opposed to favourable or neutral impact in myeloma).

Clinical features

AL amyloidosis accounts for 1 in 1500 deaths in the UK and occurs equally in men and women, equating to an incidence of about 8 per million persons per year. The median age at presentation is about 65 years, but it can occur in young adults and most likely remains underdiagnosed in the elderly.

Commonly, presenting features are non-specific, such as decreased exercise tolerance, fatigue, anorexia, weight loss and malaise. Proteinuric renal dysfunction and cardiac failure are common, and the combination is strongly suggestive of amyloidosis. The heart is affected in more than 70% of patients and in 30% restrictive cardiomyopathy is a presenting feature. Rarer cardiac presentations include arrhythmias and angina, the latter sometimes due to coronary amyloid angiopathy. Dominant renal amyloid is the presenting feature in one-third of patients, typically presenting with nephrotic syndrome and/or renal impairment. Gut involvement (either direct or more commonly due to autonomic neuropathy) causes motility disturbances, malabsorption, and less commonly, perforation, haemorrhage or obstruction. Macroglossia occurs in 5–10%, but is almost pathognomonic of AL amyloidosis (Figure 29.1). Other soft-tissue infiltration may occur, characteristically involving the submandibular region or the glenohumeral joints and surrounding tissues to produce the 'shoulder pad' sign, or lymph nodes themselves can be infiltrated. Hyposplenism is not infrequent but is rarely documented. Painful sensory polyneuropathy with changes in pain and temperature sensation followed later by motor deficits occur in 10–20% of cases and carpal tunnel syndrome occurs in 20%. Autonomic neuropathy leading to impotence, orthostatic hypotension and gastrointestinal disturbances may occur alone or together with peripheral neuropathy – a combination that should strongly raise suspicion for amyloidosis in absence of long-standing uncontrolled diabetes.

Figure 29.1 Macroglossia in AL amyloidosis.

Involvement of dermal blood vessels is common and may cause purpura, most distinctively in a periorbital distribution ('raccoon eyes'). Direct skin involvement is uncommon in systemic AL and is more frequent in localised AL. It presents as papules, nodules and plaques, usually on the face and upper trunk. Articular amyloid is rare, but the symptoms can be severe and superficially mimic an inflammatory polyarthritis. Thyroid infiltration with amyloid, occasionally resulting in hypothyroidism, has been reported. An uncommon, but serious manifestation peculiar to AL amyloidosis is an acquired bleeding diathesis that may be associated with deficiency of factor X and sometimes also factor IX.

Diagnosis and investigation of AL amyloidosis

Amyloidosis should be considered in the differential diagnosis of patients with a multisystem disease and combination such as nephrotic syndrome + restrictive cardiomyopathy, peripheral and autonomic neuropathy; acquired factor X deficiency; thick-walled heart failure with low voltage electrocardiogram and typical periorbital purpura or macroglossia in patients known to have clonal B-cell dyscrasias. In practice, the diagnosis of amyloidosis is usually an unexpected finding following biopsy of an organ with disturbed function. An approach to diagnosis is outlined in Figure 29.2, and comprising confirmation of amyloid, determination of fibril type, characterisation of the underlying plasma cell dyscrasia, and evaluation of the extent, distribution and function of involved organs.

Confirming the presence of amyloid

Histology

Histological confirmation of amyloidosis is almost always required. Critically, lack of amyloid deposits on a biopsy (any biopsy) does not categorically rule out amyloidosis due to the patch nature of the disease. Amyloid deposits occur in blood vessels and small interstitial foci throughout the body, providing the basis for 'screening' biopsies of the GI tract and abdominal fat aspiration, which are both diagnostic in 50–80% of cases. The combination of abdominal fat and bone marrow biopsy will identify amyloid deposits in 80% of cases with AL amyloidosis. Biopsy of a clinically affected organ is diagnostic in 95% of cases but can be avoided in many cases. Pink amorphous material on haematoxylin and eosin-stained tissue should raise suspicion of amyloid and prompt Congo red staining, which produces green birefringence under cross-polarised light and is the diagnostic gold standard (Figure 29.3). False-positive and false-negative interpretation of the Congo red stain is not rare but can be minimised by using the alkaline-alcohol method (described by Puchtler and colleagues), fresh reagents, tissue sections of optimal 5–10 μm thickness, inclusion of positive control tissue and high-quality polarising filters. Some Congo red techniques stain connective tissues quite strongly and produce white or very

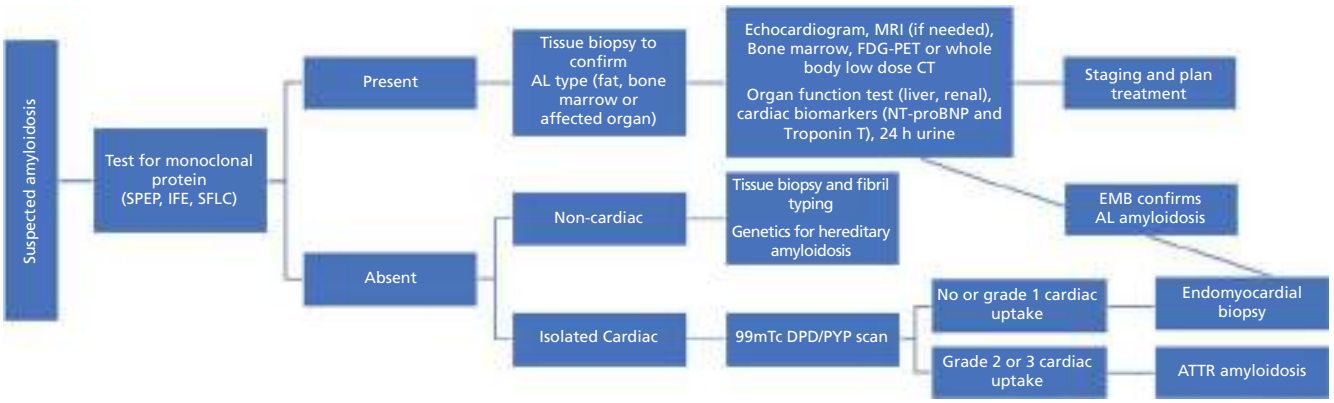


Figure 29.2 Suggested approach to the investigation of suspected amyloidosis. EMB, endomyocardial biopsy; IFE immunofixation electrophoresis; NT-proBNP N-terminal pro-brain natriuretic peptide; sFLC, serum free light chains; SPEP, serum protein electrophoresis.

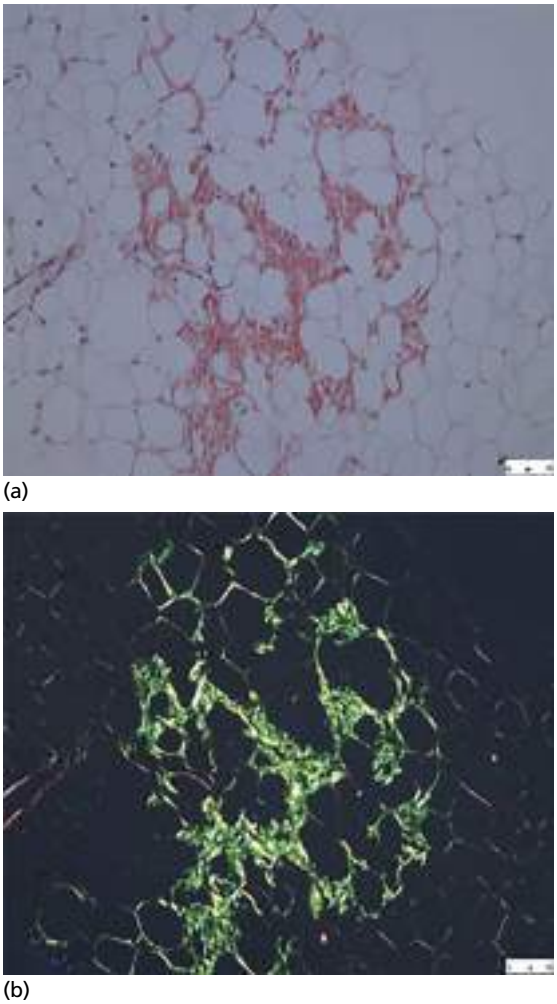


Figure 29.3 Appearance of amyloid in a bone marrow biopsy (×40, 6-μm section). (a) Congo red stain showing amorphous pink material in the interstitium and small blood vessel (arrow). (b) The same section under high-intensity cross-polarised light showing diagnostic apple-green birefringence.

pale-green birefringence that causes diagnostic errors. The differential diagnosis includes light chain deposition disease (LCDD), but these non-fibrillar deposits do not stain with Congo red.

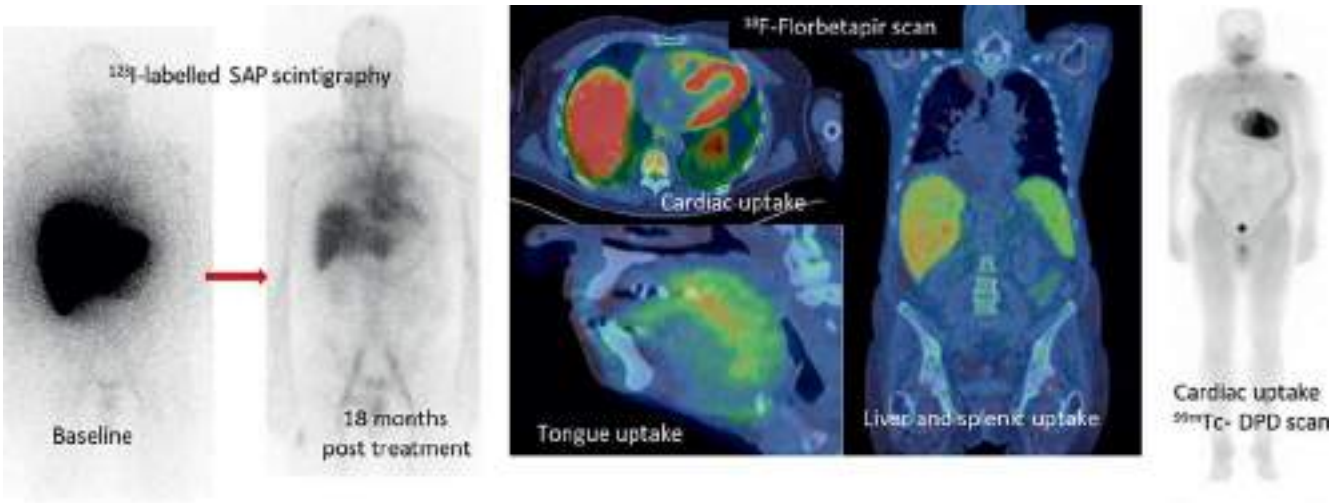
Electron microscopy is helpful in confirming the presence of amyloid and excluding LCDD. A diagnosis of amyloidosis made through electron microscopy alone needs additional clinical or imaging correlated to confirm amyloidosis.

Imaging in amyloidosis

Imaging of amyloid deposits is difficult but, when possible, may give important information about the extent of the disease. Imaging is also becoming important in assessing prognosis for patients with amyloidosis. Echocardiogram with measurement of global longitudinal strain is a widely available tool that can be used in routine clinical practice. Cardiac magnetic resonance imaging can give detailed structural information about amyloid heart and provide additional prognostic information. However, it has limitations as it cannot be done in patients with implanted devices or impaired renal function. Radionuclide imaging using ^{123}I-labelled serum amyloid P component scintigraphy can serially monitor changes in the total body amyloid load – and can act as a useful guide to therapy. However, this is not widely available but is routinely used in the UK National Amyloidosis Centre (Figure 29.4). PET scan using tracers such as Florbetpir or Florbetaben appear to be highly sensitive and specific for cardiac imaging in AL and may well become a new standard of care. Imaging with ^{99m}Tc-labelled DPD/PYP, bone scanning agents, is highly sensitive for imaging cardiac ATTR amyloidosis and useful non-invasive tool to differentiate between cardiac AL and ATTR amyloidosis (18).

Identifying fibril type

A monoclonal gammopathy may be incidental to the presence of amyloid and be gravely misleading. AL amyloidosis must be confirmed through immunohistochemical staining,



(a) (b) (c)

Figure 29.4 Radionuclide imaging in amyloidosis: (a) Radiolabelled^{123} I-SAP whole-body scintigraphy, anterior images, showing marked hepatic uptake at baseline with nearly complete regression of amyloid deposits from the liver 18 months after achieving a complete response (CR) to treatment. (b) 18F-Florbetapir scan showing cardiac, soft tissue (tongue) and hepato-splenic uptake – a new promising amyloid-specific tracer. (c) ^{99m}Tc-diphosphono-1,2-propanodicarboxylic acid (DPD) scan in a patient with wild-type cardiac transthyretin amyloidosis. There is marked cardiac uptake to the extent that the normal bone signal is not visualised.

proteomic analysis or by comprehensive exclusion of other types, including hereditary forms through DNA analysis. This is becoming critically important as elderly patients with wild type ATTR amyloidosis are diagnosed with increasing frequency and have a monoclonal gammopathy (also commoner with age) in nearly a quarter of all cases. The two important methods for fibril typing involved immunostaining with a panel of antibodies to the commoner amyloid fibril proteins (kappa, lambda, SAA, transthyretin, LECT2 and ApoA1) or laser capture of the amyloid deposit followed by tryptic digestion and mass spectrometry. The latter is now considered as the gold standard for fibril typing. Immunohistochemical staining of amyloid-containing tissue sections using a panel of antibodies should only be undertaken in experienced laboratories due to very high false positive and negative results with the exception of staining with antibodies to SAA and LECT2.

Ruling out hereditary amyloidosis

Sequencing of the genes associated with hereditary amyloidosis should be performed routinely to exclude hereditary amyloidosis when the AL type cannot be confirmed with absolute certainty by immunohistochemistry and/or proteomics. Of patients attending the UK National Amyloidosis Centre, 5% have hereditary amyloidosis, those with TTR mutations usually presenting with neuropathy and/or cardiac amyloidosis and those with fibrinogen Aα chain mutations presenting with renal dysfunction. It is necessary to confirm that identified mutations are indeed the cause of the amyloid with fibril typing as described above.

Assessment of the clone

All patients with suspected amyloidosis should have both serum and urine tested for monoclonal protein with electrophoresis and immunofixation as well as measurement of serum-free light chains. Using a combination of serum/urine plus serum-free light chains 98% of patients with AL amyloidosis will show a monoclonal component, although at a low level in many cases. All patients should have bone marrow biopsy with appropriate cytogenetic evaluation for presence of translocations or ploidy changes. Cross-sectional imaging with FDG positron emission tomography (PET) or low dose whole body CT or whole-body MRI is needed to exclude myeloma-defining bone lesions.

Assessment of organ involvement

International society of amyloidosis has developed criteria to define organ involvement in AL amyloidosis (Table 29.2). ECG and two-dimensional Doppler echocardiography are often the initial tools for evaluating cardiac involvement. In cardiac amyloidosis, ECG may demonstrate reduced QRS voltages in the limb lead and poor R-wave progression in the chest leads ('pseudo-infarct' pattern). Cardiac amyloidosis is a restrictive cardiomyopathy and diastolic dysfunction is easily missed and difficult to quantify. Echocardiography typically reveals concentrically thickened left ventricle with echogenic heart valves with impaired diastolic function. Global longitudinal strain shows early and marked impairment of the longitudinal systolic function of the heart – a very

Table 29.2 Non-invasive diagnostic criteria for amyloid-related major organ involvement.

Heart
Echocardiogram demonstrates mean wall thickness >12 mm, no other cardiac cause found
Kidney
Proteinuria >0.5 g per 24 hours, predominantly albumin
Liver
Total liver span >15 cm in the absence of heart failure or alkaline phosphatase >1.5 times the institutional upper limit of normal
Nerve
Peripheral: clinical; symmetric lower extremity sensorimotor peripheral neuropathy
Autonomic: gastric emptying disorder, pseudo-obstruction, voiding dysfunction not related to direct organ infiltration
Gastrointestinal tract
Direct biopsy verification with symptoms
Lung
Direct biopsy verification with symptoms
Interstitial radiographic pattern
Soft tissue
Tongue enlargement, clinical
Arthropathy
Claudication, presumed vascular amyloid
Skin
Myopathy by biopsy or psudohypertrophy
Lymph node (may be localised)
Carpal tunnel syndrome

(*Source:* Gertz *et al.* (2005). Reproduced with permission of John Wiley & Sons Ltd.x.)

specific feature of cardiac amyloidosis and a crucial marker for prognosis. Cardiac magnetic resonance imaging (MRI) is a very useful tool for identifying cardiac amyloidosis and distinguishing it from other restrictive cardiomyopathies and should be undertaken in all patients where the echocardiographic picture is unclear. The status of other organs can be assessed either through routine tests of organ function, such as liver function tests, serum creatinine and 24-hour urine measurements, or by specialist investigations as indicated, for example autonomic function and nerve conduction tests and high-resolution pulmonary computed tomography (CT).

Glomerular proteinuria (predominantly albuminuria) is present in about 90% of patients with renal involvement. Liver amyloidosis is often asymptomatic and presented with abnormal alkaline phosphatase and GGT as the early serological markers with other transaminases and bilirubin abnormalities seen only in very advanced disease.

Coagulation abnormalities in amyloidosis can be challenging. Nephrotic patients are often pro-thrombotic. Conversely, an abnormal clotting screen is relatively common. A prolonged thrombin time is the most frequent abnormality and may be a technical consequence of hypoalbuminaemia, and is generally of no clinical consequence. All patients with a prolonged prothrombin time or significant hepatic/splenic amyloid deposits should have formal measurement of clotting factors, particularly factor X; deficiency of which is commonest and the most likely coagulation abnormality to be associated with clinically significant bleeding.

Cardiac biomarkers (N-terminal probrain natriuretic peptide (NT-pro-BNP) and cardiac troponin) are powerful predictors of prognosis and survival after chemotherapy in cardiac amyloidosis. These have to be interpreted in context of renal function and other known cardiac disease. Significant myocardial AL amyloidosis is excluded by NT-pro-BNP concentrations below 175 ng/L. The Mayo clinic staging system incorporates NT-proBNP and troponin-T (later modification also including serum-free light chains). Stage 1, with cardiac biomarkers below threshold values (cTnT <0.035 μg/L and NT-proBNP <332 ng/L) is associated with the lowest risk of death and a median survival not reached at 5 years (90% alive at 5 years); stage 2, with either biomarker above the threshold values, has a survival 70% at 5 years; Stage 3a, with both biomarkers above the threshold values but NT-proBNP <8500 ng/L, is associated with a median survival of 36 months; and stage 3b (with NT-proBNP >8500 ng/L) has a median survival of 4 months.

Differential diagnosis

Alternative diagnoses that frequently need to be considered include amyloidosis of non-AL types (principally wild-type cardiac ATTR amyloidosis, less frequently ALECT2, AA and hereditary forms). In all cases with amyloid deposits at a single site, localised forms of AL amyloidosis become more likely. Isolated proteinuric renal involvement in presence of a monoclonal protein, raises the strong possibility of a renal lesion associated with monoclonal gammopathy of renal significance (MGRS), LCDD is the commonest. Isolated peripheral neuropathy is an uncommon presenting feature in AL amyloidosis with POEMS and other paraprotein-associated neuropathies as crucial differential diagnosis as indeed treatment-related toxicities.

Natural history

Systemic AL amyloidosis is a progressive disease with a very poor prognosis, if untreated. Median survival without treatment in historical series is only 12–15 months but has improved to over 5 years in contemporary cohorts. At diagnosis, 40% of patients have Mayo Stage III disease (elevated cardiac troponin and NT-pro-BNP) and continue to have a high early mortality

(30–40% in 12 months from diagnosis). However, amyloidosis is not, as previously believed, inexorably progressive and irreversible – it is now clear that amyloid deposits almost always gradually regress when the supply of amyloid fibril precursor protein is eliminated.

Management

Specific therapies that inhibit formation of amyloid or enhance its clearance are not yet available, although are in development. Currently, the objective of treatment in AL amyloidosis is to suppress production of amyloidogenic monoclonal light chains with the aim that progression of the disease will be slowed, halted or reversed. There has been remarkable progress in the treatment of AL amyloidosis using drugs developed for multiple myeloma with improving depth of response and decreasing mortality (Figure 29.5). In 2021, daratumumab-cyclophosphamide-bortezomib-dexamethasone (Dara-VCd) has become the first licensed treatment for AL amyloidosis.

Goals of treatment

Response in AL amyloidosis involves assessment of the direct impact of chemotherapy on the clone and, an indirect effect, of improvement in organ function on the a priori organ damage (an 'organ response') as well as depth of haematologic response achieved. Although the deeper the haematologic response, the greater is the likelihood of organ responses, there may be a discrepancy between the haematologic response and organ response. While organ response is the ultimate goal, since none of available agents can influence this directly, achieving a very good partial hematologic response (VGPR) was considered as the 'goal' of treatment in AL amyloidosis. It is clear that a CR is associated with superior outcomes and, lately, single centre studies shown that reaching very low free light chain levels after treatment (difference between involved and uninvolved light chains (dFLC) or involved free light chain [iFLC]) is also predictive of better outcomes. Thus, achieving a complete haematologic response is the goal of treatment in AL amyloidosis, ideally associated with iFLC <20 mg/L or dFLC <10 mg/L.

Haematologic response should be evaluated at least fortnightly for the initial 1–2 cycles and then monthly. Early treatment modification is imperative if the patient does not achieve progressive FLC response within the initial cycles (if the response following two cycles is ≤ partial response (PR) or following three cycles < VGPR and no organ response has occurred). An algorithm to help treatment modification based on depth of response has been published.

Certain organs affected by amyloid tend to fare better than others, following treatment. Proteinuria and liver function often improve when the clonal disease is adequately suppressed. While it was previously considered that cardiac disease would not improve, it is clear that with a CR, the majority of patients will achieve significant cardiac improvements. Improvement in macroglossia and peripheral nerve function tend to respond extremely slowly if at all. Criteria on the definitions of clonal and organ responses and progression were published by the International Society of Amyloidosis in 2012 and updated in 2020.

An approach to treatment of AL Amyloidosis

Elimination of the bone marrow clone producing the amyloidogenic light chains remains the mainstay of therapy for AL amyloidosis. Three factors determine the choice of therapy: (i) Age/performance status (ii) Extent of amyloidotic organ involvement (iii) Bone marrow plasmacytosis. Younger fit patients with limited amyloid-related end organ damage remain candidates for high-dose melphalan followed by autologous stem cell transplantation (SCT) while older/less fit patients with advanced organ involvement are treated with combination chemotherapy (but with the caveat that some may recover enough to become transplant candidates at a later date). Patients with low-level plasma cell infiltration may not need prolonged treatment courses while those with >20% plasma cells need to closely follow the paradigms established

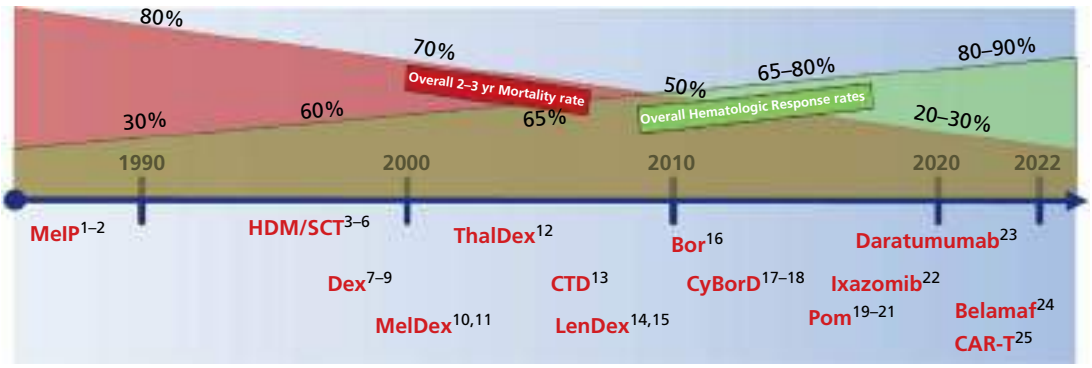


Figure 29.5 Improving response and decreasing mortality with wider access to AL treatments (*Source:* Wechalekar and Sanchorawala 2022/with permission from Elsevier).

for treatment of multiple myeloma. The International Society of Amyloidosis has published guidelines for both transplant and non-transplant chemotherapy for treatment of AL amyloidosis which should form the guiding principles for treatment choice in AL amyloidosis.

Autologous peripheral blood stem cell transplantation

Pioneered in the mid-1990's, high-dose melphalan therapy and autologous SCT was the first transformative treatment in AL amyloidosis and still the only treatment showing very durable responses lasting 10 years or more. The treatment-related mortality (TRM) in AL amyloidosis has largely been eliminated by careful and strict patient selection. Due to these criteria, only 15–20% of all patients are suitable for SCT. Advanced cardiac involvement (NT-proBNP >5000 ng/L or troponin T >0.06 ng/L), recurrent pleural effusions, poor performance status, supine systolic blood pressure <90 mm of Hg, GI bleeding and uncontrolled autonomic neuropathy are absolute contraindications for transplantation. Stem cell mobilisation has significant risks in AL amyloidosis with cardiac involvement and should be undertaken with growth factors alone. Regardless, because of its special problems in AL amyloidosis, it is recommended that such patients are treated in units with expertise in this particular disease (undertaking at least 4–5 AL transplant annually). Melphalan dose reduction in the conditioning (apart from renal impairment) leads to a significant compromise in the efficacy. Hence, dose-reduced melphalan SCT, apart from renal reasons, is not recommended in AL.

The exact role of SCT in AL amyloidosis has changed over the years. The current recommendations suggest that induction chemotherapy should be considered for the vast majority of patients with AL amyloidosis using a bortezomib-based regime. Patients with >10% bone marrow plasma cell infiltration as well as selected younger patients with even a lower plasma cell infiltrate and achieving less than a CR should proceed, as long as eligible, for SCT. There is no routine indication for SCT in patients with <10% bone marrow plasmacytosis and achieving CR with induction chemotherapy to proceed with SCT.

There is no role for allogeneic SCT in the treatment of AL amyloidosis.

Non-transplant chemotherapy for AL amyloidosis

The majority of patients with AL amyloidosis are treated with combination chemotherapy. The treatment has to be risk-adapted based on the extent of end-organ damage by amyloid deposits. Patients with advanced cardiac disease or severe autonomic neuropathy are at particular risk of treatment toxicity and may need inpatient care at the start of chemotherapy. Close and meticulous attention to supportive care is critical to success of the treatment programme.

Bortezomib has been mainstay of upfront treatment in AL for the last decade. The benefit of bortezomib-melphalan-dexamethasone was clearly demonstrated in a recent randomised phase III trial of 100 newly diagnosed patients with AL amyloidosis; improving CR/VGPR rates from 28 to 53%. The most widely used regimen is CyBorD (combination of cyclophosphamide, bortezomib and dexamethasone). A European collaborative study of 230 patients demonstrated efficacy of CyBorD, reporting haematologic, renal and cardiac response rates of 60, 25 and 17%, respectively. A study from UK National Amphoidosis Centre reported the outcomes of 915 patients with haematologic, renal and cardiac response rates of 65, 15.4 and 32.5% respectively. A rapid response with bortezomib-based therapy can significantly improve outcomes even in advanced cardiac patients (median OS improving from 5 to 26 m in patients achieving a CR/VGPR by end of 1 month).

The ANDROMEDA clinical trial compared daratumumab or placebo combined with CyBorD. In 388 patients who underwent randomisation, at a median follow-up of 11.4 months, the percentage of patients who had a haematologic CR was significantly higher in the daratumumab group than in the control group (53.3 versus 18.1%) (relative risk ratio, 2.9; 95% confidence interval [CI], 2.1–4.1; $P < 0.001$). Survival free from major organ deterioration or haematologic progression favoured the daratumumab group (hazard ratio for major organ deterioration, haematologic progression, or death, 0.58; 95% CI, 0.36–0.93; $P = 0.02$). At 6 months, more cardiac and renal responses occurred in the daratumumab group than in the control group (41.5 versus 22.2% and 53.0 versus 23.9%, respectively). The four most common grade 3 or 4 adverse events were lymphopenia (13.0% in the daratumumab group and 10.1% in the control group), pneumonia (7.8 and 4.3%, respectively), cardiac failure (6.2 and 4.8%) and diarrhoea (5.7 and 3.7%). Systemic administration-related reactions to daratumumab occurred in 7.3% of the patients. A total of 56 patients died (27 in the daratumumab group and 29 in the control group), most due to amyloidosis-related cardiomyopathy. The addition of daratumumab to bortezomib, cyclophosphamide and dexamethasone was associated with higher frequencies of haematologic CR and survival free from major organ deterioration or haematologic progression. The results of this study led to the licensing of daratumumab-CyBorD for front-line treatment of AL amyloidosis.

Alkylator-based chemotherapy regimens were the mainstay for many years but have now been superseded by combination novel agent-based chemotherapy. Oral melphalan and dexamethasone (MDex) induced a haematologic response rate of 67%, a CR rate of 33% and organ response rate of 48% in a phase II study of 45 patients. An update of this cohort with 5-year follow-up showed impressive median progression-free survival (PFS) of 3.8 years and median OS of 5.1 years. It still continues

to have a role in selected patients who are not candidates for novel agent chemotherapy.

Immunomodulatory drugs such as lenalidomide, pomalidomide and thalidomide have been used in treatment of amyloidosis for many years. Thalidomide-based treatments are no longer considered appropriate for patients with amyloidosis due to high toxicity. Lenalidomide and pomalidomide are useful for patients in treatment of relapsed disease. In phase II trials, full-dose lenalidomide had significant toxicity requiring dose reductions or discontinuation and was better tolerated at 15 mg/day in combination with weekly dexamethasone (LenDex), with haematologic response rates of 40–50%. Single-agent lenalidomide has limited efficacy and is not recommended. A retrospective study in relapsed AL treated with LenDex, there was a 49% haematologic response rate with 16% CR, median OS of about 2 years, and in those achieving CR, a PFS of over 3 years. In a preliminary report on a phase II study of pomalidomide with weekly dexamethasone, over 30% of relapsed AL patients achieved a CR, highlighting the promise of this potent immunomodulatory (IMiD) therapy. A combined retrospective analysis reported good responses (VGPR or better in 40%) with 27 months (95% confidence interval 15·7–38·1 months) and median progression-free survival (PFS) was 15 months (95% confidence interval 6·24–23·77) in multiply relapsed AL patients treated with pomalidomide.

Translocation t(11;14) is seen in 45% of patients with AL amyloidosis. This is a potential group for targeted therapy due to specific activation of cyclin D1. Venetoclax is one such agent which has been used in the treatment of multiple myeloma. We recently reported a retrospective international collaborative study showing high response rates in patients treated with Venetoclax in patients with relapsed AL amyloidosis. Amongst 43 patients reports, the haematologic response rate for all patients was 68%; 63% achieved VGPR/CR. Patients with t(11;14) had higher haematologic response (81 versus 40%) and higher VGPR/CR rate (78 versus 30%, odds ratio: 0.12, 95% CI 0.02–0.62) than non-t(11;14) patients. The toxicities appeared to be modest and the deep response rates were striking. This agent, or similar agents, needs to be investigated in prospective trials in AL (Figure 29.6).

BMPC, bone marrow plasma cells; CR, complete remission; CyBorD, cyclophosphamide-bortezomib-dexamethasone; dara, daratumumab; dFLC, difference between the involved and uninvolved free light chains; Len, lenalidomide; SCT, stem cell transplantation; VGPR, very good partial remission.

Supportive treatment

Supportive therapy remains a critical component of management of AL amyloidosis. For cardiac amyloidosis, the mainstay of treatment is diuretics. Vasodilating drugs and β-blockers are generally best avoided as they often worsen symptoms significantly. Fluid overload should be treated with fluid restriction, loop diuretics and a low-salt diet; often with addition of aldosterone antagonists (spironolactone/eplerenone). Salt-poor albumin infusions can occasionally be helpful in severely nephrotic patients. Dysrhythmias may respond to amiodarone or to pacing. Implantable cardioverter defibrillators have been used very effectively in selected patients.

In autonomic neuropathy, fludrocortisone may cause or exacerbate fluid retention. Midodrine is an effective pressor agent, starting at 2.5–15 mg thrice daily. Midodrine causes activation of the α-adrenergic receptors of the arteriolar and venous vasculature, producing an increase in vascular tone and elevation of blood pressure. Its chief adverse effect is supine hypertension, and other pressor agents must be coadministered with caution. Gastroparesis causing symptoms of early satiety and nausea can be managed with prokinetic agents such as metoclopramide, along with advice about small frequent meals that are of soft consistency. Diarrhoea due to amyloid gut involvement or autonomic neuropathy may respond to loperamide and codeine phosphate. Refractory diarrhoea can respond to octreotide. Malnutrition is not uncommon and is a poor prognostic factor. Formal and early review by an experienced dietician is important. Most patients, particularly during chemotherapy, need calorie/protein supplementation. Marked soft tissue amyloidosis involving the tongue or mouth area results in severe dysphagia and may need feeding via a percutaneous endoscopic gastrostomy. Amyloid- and treatment-related peripheral neuropathy can be disabling and difficult to treat. Amitriptyline, venlafaxine, antiepileptics, TENS and/or gabapentin/pregabalin have all been used with variable efficacy. Withdrawal or dose reduction of any neurotoxic chemotherapies, such as thalidomide or bortezomib, is needed in all such cases.

Bleeding and thrombosis are both seen in amyloidosis; and occasionally in the same patient. Patients with severe nephrotic syndrome, atrial fibrillation (or even poor atrial function) and on treatment with immunomodulatory agents need appropriate anticoagulation. Use of novel oral anticoagulants, like rivoroxaban or apixaban, appears safe in patients with good renal function. Conversely, patients with extensive gut amyloid deposits, skin amyloid deposits and those with factor X deficiency have a high risk of bleeding. Patients with factor X deficiency and clinically significant bleeding need treatment with either clotting factor concentrates (high thrombotic risk), activated factor VIIa or, if available, factor X concentrate replacement. Tranexamic acid can be useful.

Surgical resection of amyloidotic tissue is occasionally beneficial but, in general, a conservative approach to surgery, anaesthesia and other invasive procedures are advised. Should any such procedure be undertaken, meticulous attention to blood pressure and fluid balance is essential, especially in patients with renal and/or cardiac involvement. Amyloidotic tissues may heal poorly and are liable to haemorrhage.

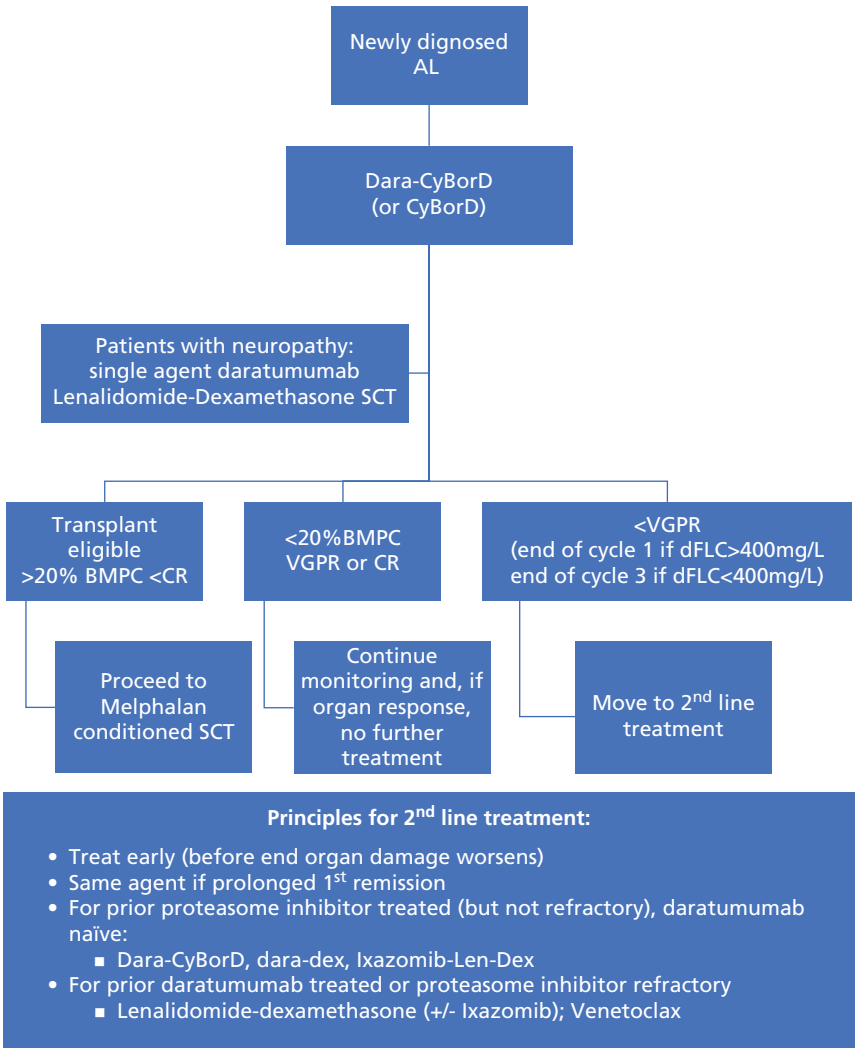


Figure 29.6 A proposed treatment algorithm for AL amyloidosis.

Solid organ transplantation

Renal and cardiac transplantation have been used in selected patients with AL amyloidosis for over 25 years. Renal transplantation should be considered in relatively young patients with otherwise well-preserved extrarenal organ function who have chemotherapy-responsive disease. Patients with renal amyloidosis transplanted when in a haematologic complete remission have good renal transplant outcomes with only rare renal grafts lost due to recurrent amyloid deposition. Cardiac transplantation can be life-saving procedure in highly selected otherwise fit patients with isolated cardiac amyloidosis but has to be followed by treatment to suppress the clone and ideally considered for SCT. Liver transplantation has been performed in only a very small number of patients, with generally disappointing results, reflecting the fact that liver failure in AL amyloidosis is always associated with substantial amyloid deposition in other vital organs.

Localised AL amyloidosis

Localised deposits of AL amyloid can occur almost anywhere in the body, with characteristic sites including the skin, airways, conjunctiva and urogenital tract. They may be nodular or confluent amyloid deposits and are associated with a usually inconspicuous focal infiltrate of clonal plasma cell producing amyloidogenic light chains. Progression of localised AL amyloid into a truly systemic disease is exceedingly rare, and conservative management is usually appropriate.

Conjunctival amyloidosis is commonest and responds to local treatment with surgical removal if symptomatic. Orbital AL amyloid presents as mass lesions that can disrupt eye movement and the structure of the orbit. These are deposits may need local or, even rarely, systemic treatment.

Airway amyloidosis may localise to larynx or involve the trachea-bronchial tree. Localised laryngeal AL amyloidosis is a well-recognised syndrome that is often amenable to direct or laser excision, but hereditary systemic apolipoprotein A-I amyloidosis can also present in this manner. Amyloidosis in the bronchial tree is virtually always of localised AL type, as are solitary or multiple amyloid nodules within the lung tissue. Patients can have significant symptoms due to airway narrowing and early bronchoscopic laser treatment of stenosing lesions can bring symptom relief. Local radiotherapy may have a role. In contrast, diffuse alveolar septal parenchymal deposition is commonly a manifestation of systemic AL amyloidosis.

Patients with Sjögren disease may present with glandular amyloid deposits – breast, salivary or lacrimal glands and lungs (either in single or multiple sites). Mucosa-associated lymphoid tissue (MALT) lymphoma should be excluded in such cases.

Lichenoid and macular forms of cutaneous amyloid are thought to be derived from keratin or related proteins. The cause is unclear and seems to be commoner in those with darker skin. Nodular cutaneous amyloid deposits are generally of AL type and can sometimes be a manifestation of systemic AL amyloidosis. Localised urogenital AL amyloid deposits are often incidental findings but may present with haematuria or, less commonly, obstruction. They can occur anywhere from the renal collecting system to the urethra, although are most usually identified within the bladder. Management is conservative or with transurethral laser resection when symptoms occur; cystectomy is rarely required.

Other forms of systemic amyloidosis

Amongst more than 5000 patients with systemic amyloidosis who have been referred for evaluation at the National Amyloidosis Centre, approximately 55% have had AL type, 15% AA (reactive systemic) type, and the remainder have had a variety of hereditary, localised and other types. Although some features of systemic AL amyloidosis are very characteristic of this particular type, such as macroglossia, periorbital purpura and certain permutations of organ involvement, the clinical phenotypes of AA, AL and hereditary systemic amyloidosis can be indistinguishable. Furthermore, the underlying chronic inflammatory disease process is clinically covert in 5–10% of patients with AA amyloidosis, and a family history is often absent in patients with hereditary ATTR and fibrinogen Aα-chain amyloidosis, which are the most common familial forms.

AA amyloidosis

Reactive systemic AA (secondary) amyloidosis occurs in 1–5% of patients with chronic inflammatory diseases that evoke a substantial acute-phase response, after a median latency of about 15–20 years. This is becoming an increasingly rare disease. AA amyloid fibrils are derived from the circulating acute-phase reactant SAA, the serum concentration of which can increase from the healthy reference range of less than 10 mg/L to over 1000 mg/L during active inflammation. The commonest associated diseases in the developed world include rheumatoid arthritis, juvenile idiopathic arthritis and Crohn's disease. Familial Mediterranean fever and other rare periodic fever syndromes can result in AA amyloidosis. Chronic infections remain important cause in some parts of the world. Castleman disease of the solitary plasma cell type should be considered amongst the underlying conditions that remain clinically covert.

Most patients present with nephropathy, particularly proteinuria, but liver and gastrointestinal involvement may occur at a late stage. Clinical involvement of the heart and nerves occurs very rarely. Treatment in AA amyloidosis should decrease the underlying inflammation so that SAA levels remain well controlled, preferably below 10 mg/L – a goal that frequently leads to the regression of existing amyloid deposits, with improvement in amyloid-related organ dysfunction, and significantly improves long-term survival. The exact nature of the treatment will depend on the underlying inflammatory disease and ranges from inhibitors of tumour necrosis factor in rheumatoid arthritis and colchicine in familial Mediterranean fever to surgical resection of Castleman disease tumours.

Leucocyte chemotactic factor II (LECT2) amyloidosis

The newly described leucocyte chemotactic factor II (LECT2) form of amyloidosis has become the second commonest acquired amyloidosis in some parts of the world. It mimics AL, AA and hereditary forms of amyloid-related kidney disease and usually manifests as isolated, stable or slowly progressive renal impairment. Asymptomatic liver involvement is not uncommon with a small proportion of patient progressing to end-stage liver failure. Diagnosis is by histological confirmation on LECT2 fibrils by immunohistochemistry or by mass spectrometry. The pathogenesis of ALECT2 amyloidosis is not known, but it is currently not thought to have a genetic aetiology. There is no known treatment for LECT2 amyloidosis apart from organ transplantation for end-stage renal disease.

Transthyretin amyloidosis (ATTR amyloidosis)

Normal *TTR* is inherently, but weakly, amyloidogenic, and minor ATTR amyloid deposits are common in elderly individuals. Clinically significant involvement is rare before the 6th decade but appears to be a relatively common occurrence in those aged over 80 years. The exact incidence remains unknown but may account for ~15% of patients with the

syndrome of heart failure with preserved ejection fraction (this estimation would suggest ~60,000 patients in the UK). Wild-type ATTR amyloidosis typically causes heart failure, carpal tunnel syndrome, spinal canal stenosis and biceps tendon rupture. Patients typically survive for several years with reasonably good quality of life managed with diuretics alone. The re-purposed use of bone tracer ^{99m}Tc-DPD or similar agents is highly sensitive for cardiac ATR amyloidosis and has greatly improved the diagnostic yield. Tafamidis is a TTR-stabilising small molecule that is licensed for this condition and has been shown to prolong survival.

Familial amyloidotic polyneuropathy (FAP) associated with mutations in the gene for *TTR* is the most common type of hereditary amyloidosis. It is characterised by progressive and disabling peripheral and autonomic neuropathy, often with cardiac involvement. Vitreous amyloid deposits may also occur and are virtually pathognomonic of the syndrome. Symptoms typically present between the third and seventh decades. More than 100 *TTR* variants are associated with FAP, the most frequent of which is the substitution of methionine for valine at residue 30 (TTR Met30). There are well-recognised foci of this in Portugal, Japan and Sweden, but FAP has been reported in most ethnic groups. *TTR* Ala60 is the most frequent cause of FAP in the British and Irish population, typically presenting after 50 years of age and usually with marked cardiac involvement. *TTR* Ile122 occurs in 3–4% of black Africans and is associated with a phenotype indistinguishable from senile (wild-type) cardiac amyloidosis. Patisiran (a small interfering RNA) and Inotersan (an antisense oligonucleotide) specifically inhibit TTR protein production and are licensed for treatment of hereditary ATTR amyloidosis with neuropathy.

Hereditary systemic amyloidoses

Hereditary systemic amyloidosis is caused by deposition of genetically variant proteins as amyloid fibrils, and is associated with mutations in the genes for *TTR*, fibrinogen Aα chain, cystatin C, gelsolin, apolipoprotein A-I, apolipoprotein A-II and lysozyme. These disorders are all inherited in an autosomal dominant manner with variable penetrance, and usually present in adult life.

The syndrome of non-neuropathic hereditary systemic amyloidosis is caused by mutations in the genes for fibrinogen Aα chain, lysozyme, apolipoprotein A-I and apolipoprotein A-II. Most such patients present with renal impairment and/or proteinuria, but substantial deposits in the liver and spleen are frequent in hereditary lysozyme and apolipoprotein A-I amyloidosis, and the heart may be involved in hereditary apolipoprotein A-I amyloidosis. Prominent neuropathy occurs in some patients with apolipoprotein A-I Arg26. Although kindreds with hereditary amyloidosis are rare, 5–10% of patients referred to the National Amyloidosis Centre with apparently sporadic amyloidosis do in fact have hereditary forms of the disease. About half of these are associated with *TTR* mutations, and most of the remainder are associated with variant fibrinogen Aα chain Val526. Penetrance of this particular mutation is extremely low in most families, thus obscuring the genetic aetiology, but the renal histology is characteristic, showing substantial accumulation of amyloid within enlarged glomeruli, but none in blood vessels or the interstitium. Renal impairment is often noted in the fourth or fifth decade, and end-stage renal failure is managed with dialysis or renal transplantation. Variant fibrinogen Aα-chain amyloidosis can recur in the transplanted graft and median survival of kidney transplants in patients attending our centre has been 7 years, though this can be much longer. Combined liver–kidney transplants have been performed in this condition though, with notable mortality. Hereditary cystatin C amyloidosis manifests in Icelandic families as cerebral amyloid angiopathy, with recurrent cerebral haemorrhage, and gelsolin variants are associated with corneal lattice dystrophy and cranial neuropathy, most often in Finnish patients.

DNA analysis should now be performed routinely on patients with systemic amyloidosis in whom AA or AL fibril type cannot be definitively verified.

Conclusion and future directions

Improved understanding of the aetiology and pathogenesis of amyloid has led to many recent advances in the characterisation and management of amyloidosis. Use of mass spectrometry has allowed for accurate amyloid typing. The clonal biology in AL shows the differences between AL and MGUS/Myeloma with potential specific targets for treatment. Imaging in amyloidosis with development of cardiac MRI and amyloid-specific tracers has been brought to the mainstream. Clinical improvement following successful treatment of the various conditions that underlie amyloidosis is always gradual, and the crucial need for multidisciplinary supportive measures is better recognised. The biggest leap has been in treatment for AL with licensing of daraCyborD as the 1st licensed treatment in 2021. The expanding therapeutic armamentarium in agents treating multiple myeloma has started making earlier inroads into amyloidosis. Active immunotherapy holds potential promise of long-term responses and may challenge the place of SCT in the treatment pathway.

Novel therapies specifically for amyloidosis now in development include small molecules, monoclonal antibodies, peptides and GAG analogues that variously interfere with amyloidogenesis. Immunotherapy approaches are in the advanced phases of clinical trials with the aim of promoting regression of amyloid now tantalisingly close. Amyloidosis, no longer a true rarity, is now a treatable condition from which patients can hope for recovery and longevity.

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PART IV

Stem cell and myeloid disorders

CHAPTER 30

Inherited aplastic anaemia/bone marrow failure syndromes

30

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Introduction

A number of inherited (constitutional/genetic) disorders are characterised by aplastic anaemia (AA)/bone marrow failure (BMF), usually in association with one or more somatic abnormalities (Table 30.1). The features of some of these are summarised in Table 30.2. The precise incidence/prevalence of these remains unclear but, collectively, they represent approximately 20–30% of patients presenting with AA and constitute a significant clinical burden, as many are associated with premature mortality. The BMF may present at birth or at a variable time thereafter, including in adulthood in some cases. The BMF may involve all lineages or a single lineage; in some cases, it may initially be associated with a single cytopenia and then progress to pancytopenia. Scientifically, they constitute an important group of diseases as advances in understanding the genetics of some of these are not only unravelling their pathophysiology, but are also providing important insights into normal haemopoiesis.

The two syndromes that are frequently associated with generalised BMF/AA are Fanconi anaemia (FA) and dyskeratosis congenita (DC). These two syndromes are also two of the best characterised and are discussed in some detail in this chapter (followed by sections on Shwachman–Diamond syndrome, Diamond–Blackfan anaemia, congenital dyserythropoietic anaemia, congenital neutropenia, thrombocytopenia with absent radii, congenital amegakaryocytic thrombocytopenia and newly identified subtypes). This chapter discusses the clinical and genetic heterogeneity of these BMF syndromes, their management and their impact on our understanding of the pathophysiology of more common forms of 'idiopathic aplastic anaemia'. Indeed, patients with FA, DC and other inherited BMF subtypes can sometimes present with AA/BMF alone as their initial manifestation and pose a diagnostic/management challenge.

Fanconi anaemia

Clinical features

Since the first description by Guido Fanconi in 1927, FA has come to be recognised as an autosomal recessive (AR) disorder (X-linked in a rare subset) in which there is progressive BMF and an increased predisposition to malignancy, especially acute myeloid leukaemia (AML). Most, but not all, affected individuals also have one or more somatic abnormalities, including skin (café-au-lait spots), skeletal (absent thumbs, radial hypoplasia, scoliosis), genitourinary (underdeveloped gonads, horseshoe kidneys), gastrointestinal, cardiac and neurological anomalies (Table 30.3). Some of these somatic abnormalities are shown in Figure 30.1. The course of the disease and the pattern of somatic abnormalities show considerable variation, with approximately one-third of patients having no physical abnormalities. This makes diagnosis based on clinical criteria alone difficult and unreliable.

The cumulative incidence of BMF by the age of 40 years in patients with FA is ~90%. At birth, the blood count is usually normal. Pancytopenia develops insidiously and presents in most cases between the ages of 5 and 10 years (median age 7 years).

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 30.1 The inherited BMF syndromes.

Pancytopenia (usually associated with a global haemopoietic defect)
Fanconi anaemia
Dyskeratosis congenita
Shwachman–Diamond syndrome
Reticular dysgenesis
Pearson syndrome
Familial aplastic anaemia (autosomal and X-linked forms) including newly characterised subtypes
Myelodysplasia
Non-haematological syndromes (Down, Dubowitz syndromes)
Single cytopenia (usually)
Anaemia
Diamond–Blackfan anaemia
Congenital dyserythropoietic anaemia
Neutropenia
Severe congenital neutropenia including Kostmann syndrome
Thrombocytopenia
Congenital amegakaryocytic thrombocytopenia
Amegakaryocytic thrombocytopenia with absent radii

Table 30.3 Somatic abnormalities in FA.

Abnormality	Percentage of patients
Skeletal (radial ray, vertebral, scoliosis andrib)	71
Skin pigmentation (café-au-lait, hyper- and hypopigmentation)	64
Short stature	63
Eyes (microphthalmia)	38
Renal and urinary tract	34
Male genital	20
Mental retardation	16
Gastrointestinal (e.g. anorectal and duodenal atresia)	14
Heart	13
Hearing	11
Central nervous system (e.g. hydrocephalus and septum pellucidum)	8
No abnormalities	30

(*Source:* Auerbach *et al.* (2001) Fanconi anaemia. In: *The Metabolic and Molecular Basis of Inherited Disease.* Adapted with permission of McGraw-Hill.)

However, in some cases, the pancytopenia develops in adolescence or even in adult life. The haemoglobin and platelet count are usually first to fall; the granulocytes are usually well-preserved in the early stages. As the pancytopenia develops, the bone marrow (BM) becomes progressively hypocellular. There is often a marked increase in macrophage activity with evidence of haemophagocytosis. BMF, leading to fatal haemorrhage or infection, is the main cause of death. In an analysis of the International Fanconi Anaemia Registry (IFAR), the median survival time was 24 years; this is changing with improvements in clinical care.

Table 30.2 Characteristics of the BMF syndromes.

	FA	DC	SDS	DBA	CDA	TAR+ CAMT	SCN	IAA	Other/New‡
Inheritance pattern	AR, XLR	XLR, AR, AD	AR	AD, XLR	AR, AD	AR	AD, AR	?	AD and AR
Somatic abnormalities	Yes	Yes	Yes	Yes	Rare	Yes	Rare	?None	Yes
Bone marrow failure	AA (>90%)	AA (~80%)	AA (20%)	RCA	Eryth	Megs	Neutropenia	Yes (100%)	Yes
Short telomeres	Yes	Yes	Yes*	?	?	?	?	Yes	No
Malignancy	Yes	Yes	Yes	Yes	?No	?No	Yes	Yes	?
Chromosome instability	Yes	Yes	Yes	?	?	?	?	Yes	?
Genes identified	22	20	4	21	5	4	7+	†	25+

* Telomeres are usually very short in DC, short in FA and SDS.
† Heterozygous variants in *TERC* and *TERT* are risk factors for some cases of AA. AA, aplastic anaemia; AD, autosomal dominant; AR, autosomal recessive; CDA, congenital dyserythropoietic anaemia; DBA, Diamond–Blackfan anaemia; DC, dyskeratosis congenita; FA, Fanconi anaemia; IAA, idiopathic aplastic anaemia; Eryth, ineffective erythropoiesis; Megs, low megakaryocytes; RCA, red cell aplasia; SCN, severe congenital neutropenia; SDS, Shwachman–Diamond syndrome; TAR, thrombocytopenia with absent radii; CAMT, congenital amegakaryocytic thrombocytopenia and other syndromic thrombocytoenias. XLR, X-linked recessive.
‡ Patients in whom genetic defects have been identified (e g. in *SRP72*, *ERCC6L2 and GATA2*) but do not fit into previously recognised categories.

Figure 30.1 FA. (a) Photographs of patients with FA (A1–A3) with small mouth and chin ('Fanconi facies'). (b) Abnormalities of pigmentation (hyper- and hypopigmentation) on the abdomen (B1) with a close-up (B2) of a café-au-lait spot and a hypopigmented patch. The bottom photograph (B3) shows the back of an FA patient demonstrating lumbar scoliosis. (c) Hands/forearms of FA children showing hypoplastic thumbs (C1), rudimentary ('dangling') thumbs (C2) and a radiograph (C3) showing rudimentary thumb (skeletal) development.

FA is associated with an increased risk of leukaemia and other malignancies. The leukaemias are usually of the acute myeloid type, particularly FAB types M4 and M5 (see Chapter 32). In some rare cases, leukaemia may be the initial event leading to the diagnosis of FA. The cumulative incidence of haematological malignancy by the age of 40 years is 33%. Recent studies suggest leukaemia development is frequently associated with chromosome 1q/MDM4 trisomy which leads to downregulation of p53 signalling. Besides haematological malignancies, there is a significant risk of hepatic tumours and squamous cell carcinoma of the vulva, oesophagus, head and neck. The cumulative incidence of solid tumours is calculated to be 28% by the age of 40 years. Malignancies occur mainly in patients with late-onset BMF and longer survival, with a median age of 13 years for leukaemia and 25 years for solid tumours. Analysis from the German FA registry largely concurs with these earlier observations. Furthermore, long-term follow-up in FA patients who have been treated by haemopoietic stem cell transplantation (HSCT) is showing a higher incidence of non-haematological malignancies in patients with FA than patients with other types of BMF who underwent HSCT.

Cell and molecular biology

Over the last four decades, many advances have been made in our understanding of the pathophysiology of FA. FA cells characteristically display a high frequency of spontaneous chromosomal breakage and hypersensitivity to DNA cross-linking agents such as diepoxybutane (DEB) and mitomycin C (MMC). This genomic instability (Figure 30.2) led to the development of a diagnostic test (i.e. increased chromosomal breakage in FA cells compared with normal controls after exposure to DEB/MMC) over four decades ago and this remains a useful FA screening test today. This 'FA cell phenotype' has also facilitated many advances in our understanding of FA, including elucidation of the complex genetics of this disease, with 22 subtypes/complementation groups currently characterised. The genes responsible for these subtypes are designated *FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM, FANCN, FANCO*, *FANCP*, *FANCQ, FANCR, FANCS, FANCT, FANCU, FANCV* and *FANCW*. (Table 30.4).

Studies from several groups have demonstrated that the proteins encoded by the FA genes participate in a complicated network important in DNA repair. Eight of the FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) interact with each other and form the FA core complex (Figure 30.3). The FA core complex is required for the activation of the FANCI-FANCD2 protein complex to a monoubiquitinated form (FANCI-FANCD2-Ub). FANCI-FANCD2-Ub then interacts with DNA repair proteins (including BRCA2, BRCA1 and RAD51) leading to repair of the DNA damage. FA-D1 patients have biallelic variants in BRCA2. These observations have linked the FA proteins with BRCA1 and BRCA2 (FANCD1) in a DNA damage response pathway 'The FA/BRCA pathway'. The BRCA2 protein is important in the repair of DNA damage by homologous recombination. Cells lacking BRCA2 inaccurately repair damaged DNA and are hypersensitive to DNA cross-linking agents. It has been established that FANCJ is BRIP1 (partner of BRCA1), FANCN is PALB2 (partner of BRCA2), and that SLX4 is also an FA protein. These findings further strengthen the connection between FA and DNA repair; specifically, it appears that the FA pathway orchestrates incisions at sites of cross-linked DNA. Recent studies suggest the FA proteins may be important in counteracting aldehyde-induced genotoxicity in haemopoietic stem cells.

The FA–BRCA pathway is activated in response to DNA damage (e.g. replication fork arrest) and involves ATR (ataxia telangiectasia and RAD3-related protein) (Figure 30.3). The pathway is inactivated by the de-ubiquitinating enzyme USP1. ATR appears to directly regulate the FA pathway as it is required for the monoubiquitination of FANCD2 and FANCI. ATR is

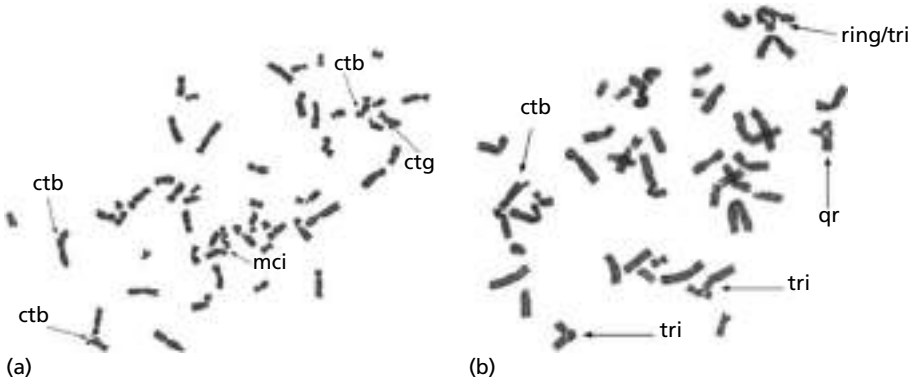


Figure 30.2 (a, b) Chromosomal abnormalities seen in FA lymphocytes following incubation with diepoxybutane. ctb, chromatid break; ctg, chromatid gap; mci, multiple chromatid interchanges (complex rearrangement); tri, triradial; qr, quadriradial (Source: Nicola Foot, Hammersmith Hospital. Reproduced with permission.)

Table 30.4 FA complementation groups/genetic subtypes.

Complementation group/gene	Approximate percentage of FA patients	Chromosome location	Exons
A (*FANCA*)	65	16q24.3	44
B (*FANCB*)	<1	Xp22.2	17
C (*FANCC*)	12	9q22.3	22
D1 (*FANCD1*)	<1	13q13.1	27
D2 (*FANCD2*)	<1	3p25.3	45
E (*FANCE*)	4	6p21.31	10
F (*FANCF*)	4	11p14.3	1
G (*FANCG*)	12	9p13.3	14
I (*FANCI*)	<1	15q26.1	38
J (*FANCJ/BRIP1*)	<5	17q23.2	25
L (*FANCL*)	<1	2p16.1	14
M (*FANCM*)*	<1	14q21.2	25
N (*FANCN/PALB2*	<1	16p12.2	14
O (*FANCO/RAD51C*)*	<1	17q22	12
P (*FANCP/SLX4*)	2	16p13.3	17
Q (*FANCQ/ERCC4*)	<1	16p13.12	13
R (*FANCR/RAD51*)*	<1	15q15.1	13
S (*FANCS/BRCA1*)*	<1	17q21.31	24
T (*FANCT/UBE2T*)	<1	1q32.1	7
U (*FANCU/XRCC2*)	<1	7q36.1	3
V (*FANCV/REV7*)	<1	1p36.22	10
W (*FANCW/RFWD3*)	<1	16p23.1	18

FA subtypes (complementation groups) A, C and G account for the majority of FA patients. As can be noted from the table, many FA genes encode proteins that had previously been known by other names and have important roles in DNA repair.
* Biallelic variants in *FANCM*, *FANCO* and *FANCS* and heterozygous variants in *FANCR/RAD51* produce FA-like disease.

mutated in a subset of patients with Seckel syndrome, a disease exhibiting some clinical similarity to FA. ATR and ATM (ataxia telangiectasia mutated) are known to phosphorylate FANCD2 and FANCI. Interestingly, ATR-Seckel cells also exhibit defects in FANCD2 monoubiquitination. Furthermore, Nijmegen breakage syndrome (NBS) cells (mutated in *NBN*) show defects in FANCD2 monoubiquitination. This suggests that as well as clinical overlap between patients with FA, NBS and Seckel syndrome, there is also overlap in the biological defects observed in cells from these patients. This highlights the complexity of physical interactions between the different molecules involved in this matrix of pathways. Equally, it is clear that FA cells also display other abnormalities in addition to DNA repair (Figure 30.3). This includes hypersensitivity to oxygen, accelerated telomere shortening, abnormal cell cycle kinetics, upregulation of p53 and over-activation of the mitogen-activated protein kinase (MAPK) pathways leading to over-production of tumour necrosis factor (TNF)-α. These observations highlight the multiple biological functions of the FA proteins.

It is noteworthy that the phenotypes associated with biallelic *BRCA2* (FA-D1) and *PALB2* (FA-N) variants are markedly similar to each other, but different from the other FA genes. Specifically, FA-D1 and FA-N are associated with high risks of solid childhood malignancies (e.g. Wilm tumour and medulloblastoma), which are not usually seen in the other FA subtypes. Furthermore, heterozygous variants in *BRCA2* (FA-D1), *PALB2* (FA-N) and *BRIP1* (FA-J) confer an elevated risk of breast cancer yet this is not the case for the other FA genes. These differences highlight that the relationship between the FA proteins and their interactions with other molecules is complex at both the clinical and molecular level.

In vitro gene transfer studies have demonstrated that introduction of the appropriate wild-type FA gene into FA human lymphoid and haemopoietic cells markedly enhances their growth and normalises their response to MMC; in lymphoid lines, cell kinetics (G_2 phase) and chromosomal breakage are normalised. Thus, the transfer of the wild-type FA genes corrects the extreme sensitivity to DNA cross-linking agents, the hallmark of the FA cell phenotype. These studies provide the rationale for haemopoietic gene therapy (discussed below).

Murine models of FA have shown that haemopoietic progenitors are hypersensitive to TNF-α and interferon (IFN)-γ. This differential hypersensitivity to IFN-γ is thought to be mediated by *fas*-induced apoptosis and may

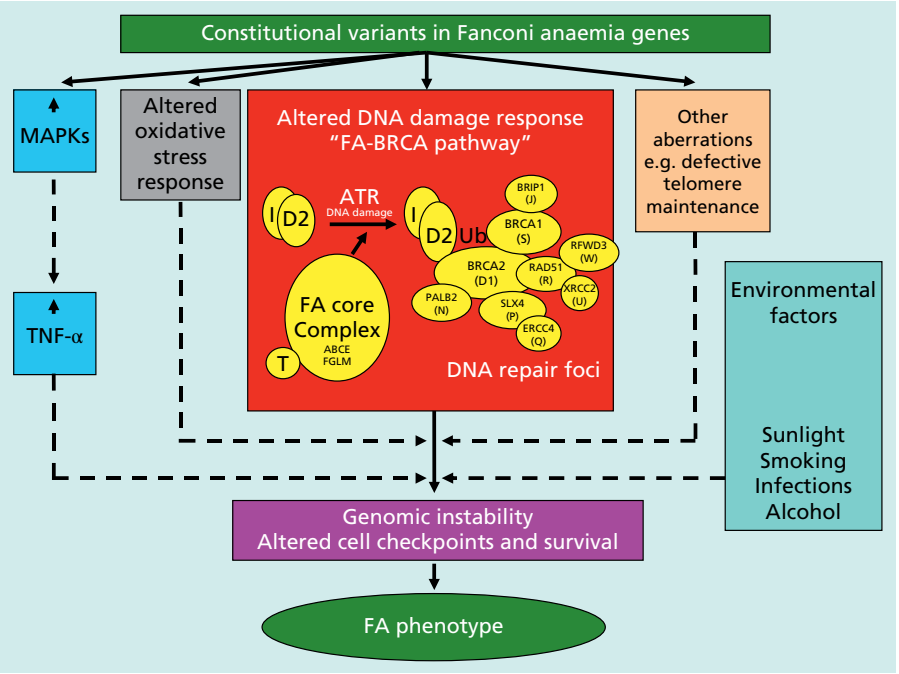


Figure 30.3 Schematic representation of the FA–BRCA pathway and related networks. The diagram shows that the constitutional variants in FA genes lead to aberration of the FA–BRCA pathway, abnormal handling of oxidative stress, aberrant activation of mitogen-activated protein kinases (MAPKs) and defective telomere maintenance, as well as other biological aberrations. The net impact of these is increased genomic instability and altered cell survival/checkpoints. The diagram also highlights the potential role of environmental factors such as smoking in adding to the effect of the constitutional FA gene variants. Within the FA–BRCA pathway, the proteins shown in yellow are those mutated in different FA patients. The FA core complex consists of eight FA-proteins (A, B, C, E, F, G, L and M), and this, together with ATR (ataxia telangiectasia and RAD3-related protein), is essential for activation (ubiquitination) of the I–D2 complex after DNA damage. Activated I–D2-Ub translocates to DNA repair foci where it associates with other DNA damage response proteins including BRCA2, RAD51 and SLX4 and participates in DNA repair. TNF, tumour necrosis factor; Ub, ubiquitination.

turn out to be an important mechanism in the development of progressive BMF in FA. It is noteworthy that patients with idiopathic AA usually have raised IFN-γ levels, thus providing a possible link in the pathophysiology of BMF in both idiopathic and FA-associated AA. The presence of short telomeres in cells from patients with both FA and idiopathic AA can also be expected to be important in the pathophysiology of BMF in both diseases. This feature is discussed further in relation to DC.

Treatment

The major cause of mortality in FA is the development of BMF. Anabolic steroids such as oxymetholone and danazol can produce useful trilineage haematological responses in 50–70% of patients but many will become refractory after a variable time. These can be associated with side-effects such as liver dysfunction; patients therefore need close monitoring. HSCT is the only curative treatment for BMF. From the *in vitro* and *in vivo* studies, it has become clear that cells from FA patients are hypersensitive to agents such as cyclophosphamide and irradiation compared with non-FA patients. Therefore, HSCT conditioning regimens have been modified by reducing the dose of cyclophosphamide and radiation. Initially, using low-dose cyclophosphamide (20 mg/kg) and 4.5–6 Gy of thoracoabdominal irradiation, the actuarial survival for patients transplanted using HLA-identical sibling donors was ~70% at 2 years. The results using unrelated donors were less good, with 2-year survival between 20 and 40%. Long-term follow-up of patients who have survived after HSCT shows a much higher incidence of malignancies (compared to idiopathic BMF), particularly of the head and neck, usually 8–10 years after the transplant. This partly relates to the inherent predisposition of FA patients to malignancy (which can perhaps now be explained given the link to defects in DNA repair) and partly to factors such as the use of radiotherapy in the conditioning. Transplant groups are therefore exploring low-dose cyclophosphamide (20–40 mg/kg) HSCT

protocols that avoid the use of radiotherapy. Results using fludarabine (120–150 mg/m^2) in association with low-dose cyclophosphamide are very encouraging for both sibling and unrelated HSCT. Longer follow-up is necessary to determine if such protocols will be associated with a lower risk of malignancy.

In addition to HSCT, alternative treatment strategies are being explored. The identification of the FA genes, combined with the *in vitro* gene transfer data, which show that FA haemopoietic stem cells rescued by gene therapy should have a selective growth advantage within the hypoplastic BM environment, has resulted in studies aimed at developing gene therapy for FA patients. Recently there has been some success in using this approach in FA-A patients.

The identification of FA *mosaic* patients strengthens the case for future trials of gene therapy. In such cases, the DEB/MMC test may be negative or only demonstrate chromosomal instability in a subgroup of cells. Somatic mosaicism is due to reversion of a pathogenic allele to 'wild' type in a single haemopoietic (somatic) cell. The mechanism of how this occurs can vary, but in each case, it generates one 'normal' FA allele and the resulting cell effectively becomes a 'heterozygous cell', which would be expected to have a growth/survival advantage in the background of FA cells. These mosaic patients can have an improvement in their haematological profile, suggesting that a single pluripotent stem cell may be sufficient to restore adequate haemopoiesis. FA patients with somatic mosaicism can thus be regarded as having undergone natural haemopoietic gene therapy.

Over the last 20 years, there has been significant progress in treating the BMF associated with FA. In future, the major challenge in Fanconi anaemia may relate to the treatment of the associated malignancies and the management of complications (e.g. pulmonary disease) in adulthood.

Dyskeratosis congenita

Clinical features

Classical DC is characterised by the mucocutaneous triad of abnormal skin pigmentation, nail dystrophy and mucosal leucoplakia (Figure 30.4). Since its first description by Jacobi in 1906 and Zinsser in 1910, a variety of non-cutaneous (dental, gastrointestinal, genitourinary, haematological, neurological, ophthalmic, pulmonary and skeletal) abnormalities have also been reported (Table 30.5). BMF is the principal cause of early mortality, with an additional predisposition to malignancy (haematological and non-haematological) and fatal pulmonary complications. X-linked recessive, AD and AR forms of the disease are recognised. DC is a very heterogeneous disorder, both clinically and genetically.

Clinical manifestations in DC often appear during childhood. The skin pigmentation and nail changes typically appear first, usually by the age of 10 years. BMF usually develops below the age of 20 years; 80–90% of patients will have developed BM abnormalities by the age of 30 years. In some cases, the BM abnormalities may appear before the mucocutaneous

Figure 30.4 Photographs of patients with DC showing abnormal skin pigmentation (a, b, c), nail dystrophy (d, e) and leucoplakia of the tongue (f).

Table 30.5 Somatic abnormalities in dyskeratosis congenita.

Abnormality	Percentage of patients
Abnormal skin pigmentation	89
Nail dystrophy	88
BMF	85.5
Leucoplakia	78
Epiphora	30.5
Learning difficulties/developmental delay/ mental retardation	25.4
Pulmonary disease	20.3
Short stature	19.5
Extensive dental caries/loss	16.9
Oesophageal stricture	16.9
Premature hair loss/greying/sparse eyelashes	16.1
Hyperhidrosis	15.3
Malignancy	9.8
Intrauterine growth retardation	7.6
Liver disease/peptic ulceration/enteropathy	7.3
Ataxia/cerebellar hypoplasia	6.8
Hypogonadism/undescended testes	5.9
Microcephaly	5.9
Urethral stricture/phimosis	5.1
Osteoporosis/aseptic necrosis/scoliosis	5.1
Deafness	0.8

manifestations and the patients may be categorised as having 'idiopathic aplastic anaemia'. The main causes of death are BMF/immunodeficiency (~60–70%), pulmonary complications (~10–15%) and malignancy (~10%).

The minimal clinical criteria for diagnosis of DC includes the presence of at least two out of the four major features (abnormal skin pigmentation, nail dystrophy, leucoplakia and BMF) and two or more of the other somatic features known to occur in DC.

Cell biology and link to other diseases

Since 1998, 20 DC genes have been identified and these account for ~80% of DC (Table 30.6).

X-linked DC and the Hoyeraal-Hreidarsson (HH) syndrome

The gene (*DKC1*) responsible for X-linked DC was mapped to Xq28 in 1986 and identified through positional cloning in 1998. The *DKC1* gene is highly conserved and encodes the protein dyskerin. With the identification of variants in *DKC1* the first diagnostic tests became available. It also provided the first firm evidence that DC was not a homogeneous disorder and that other syndromes with overlapping presentation can share the same genetic mutations. The first such example was the HH syndrome, a severe multisystem disorder that is characterised by intrauterine growth restriction, microcephaly, cerebellar hypoplasia, AA and immunedeficiency. Due to the overlap in features it was suggested and subsequently proven that HH is a severe variant of DC due to the presence of *DKC1* variants in males with the classical presentation of HH. However, *DKC1* variants are not the only cause of HH; variants in other genes can also lead to a phenotype of HH (see below).

Autosomal dominant (AD)-DC and its link to telomeres and other diseases

AD-DC is heterogeneous; heterozygous variants in several genes (including *TERC*, *TERT* and *TINF2*) have been characterised. The identification of heterozygous variants in *TERC* (telomerase RNA component) in 2001 was a major advance in the DC field, as it provided a direct link between DC and telomerase. Telomerase is a ribonucleoprotein composed of two core components: a catalytic component that adds the repeats, telomerase reverse transcriptase (*TERT*) and *TERC*, which acts as the template. It functions as a specialised polymerase that adds the telomeric repeat (TTAGGG) to the end of the 3′ lagging strand of DNA after replication. Due to the semi-conservative nature of DNA replication, telomerase is essential to maintain telomere length in rapidly dividing cells, as found in the haemopoietic system. Without telomerase the telomeres shorten with each successive round of replication, when they reach a critical length, the cells enter senescence. Telomerase is mainly restricted to cells such as germ cells, stem cells and their immediate progeny, activated T cells and monocytes, but in cells where telomerase is not present telomere shortening is part of the normal process of cellular ageing.

In patients with AD-DC variants were identified in *TERC* initially and it was through the identification of variants within this molecule that led to significant expansion of the DC phenotype to include other haematological and non-haematological disorders. Firstly, heterozygous *TERC* variants were identified in patients with AA and soon after in patients with myelodysplastic syndrome (MDS).

The identification of constitutional variants in *DKC1* and *TERC* established the pathology of defective telomere maintenance (Figure 30.5) as being the principal underlying cause of DC; both dyskerin (encoded by *DKC1*) and *TERC* are now recognised to be core components of telomerase and patients with *DKC1* and *TERC* variants have very short telomeres compared to age-matched controls. This led to further study of the telomerase complex to determine the genetic basis of the remaining uncharacterised patients. The next gene to have variants identified was *TERT*, which encodes the enzymatic component of the telomerase complex. The clinical presentation in patients with *TERT* variants is highly variable, ranging from near DC phenotype to just AA. Heterozygous variants in *TERT* and *TERC* have

Table 30.6 Genetic subtypes of dyskeratosis congenita.

DC subtype	Approximate percentage of patients	Chromosome location	RNA/protein product	Exons
X-linked recessive	25	Xq28	Dyskerin	15
Autosomal dominant	12	14q11	TIN2	6
	5	3q26.2	TERC*	1
	3	5p15.33	TERT*	16
	<1	4q32.2	NAF1*	13
	<1	12q24.31	ZCCHC8*	17
	<1	5q35.1	NPM1	13
	<1	16q22.1	TPP1	12
	<1	20q13.3	RTEL1*	35
	<1	17p13.3	RPA1	17
	<1	1q32.1	MDM4	13
	<1	7q31.33	POT1*	21
Autosomal recessive	2	16q21	USB1	9
	2	18p11.32	TYMS-ENOSF1	8 and 22
	2	20q13.3	RTEL1*	35
	2	16p13.12	PARN*	27
	<1	15q14	NOP10	2
	<1	5p15.33	TERT*	16
	<1	5q35.3	NHP2	4
	<1	17p13.1	WRAP53/TCAB1	13
	<1	17p13.1	CTC1	23
	<1	16q22.1	ACD/TPP1	12
	<1	1p13.2	DCLRE1B	5
Uncharacterised	>20	?	?	?

*Heterozygous variants in these genes have been associated with pulmonary disease in late adulthood. The majority of the DC genes encode products that have a principal role in telomere maintenance; however, this is not the case for USB1 and NPM1.

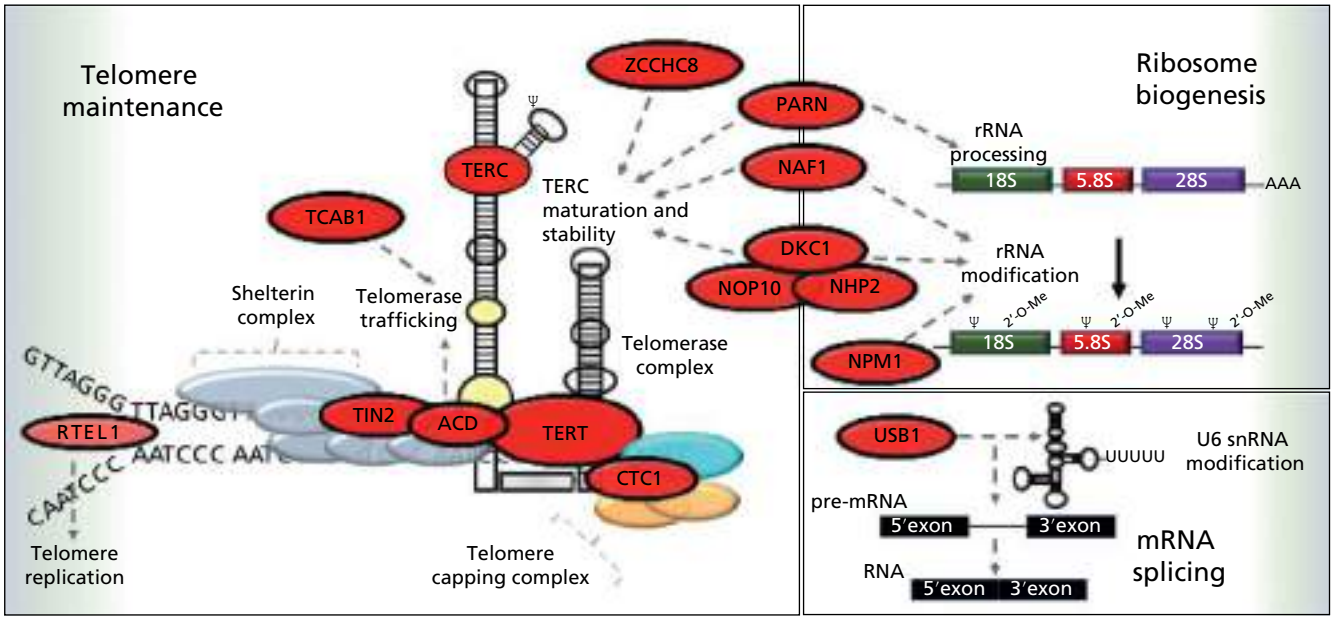


Figure 30.5 Functional overlap of DC genes involved in telomere maintenance and ribosome biogenesis. Proteins mutated in DC are indicated by red capsules and affect molecular functions such as telomere replication (RTEL1), telomere protection (TIN2, ACD) telomerase (TERC and TERT and DKC1), telomerase maturation and stability (ZCCHC8, NAF1, PARN, DKC1, NOP10 and NHP2). Pseudouridylation of TERC and ribosomal RNA (rRNA) is performed by DKC1. The deadenylation function of PARN also regulates the maturation and processing of both TERC and rRNA. Recently, variants in NPM1 that regulate 2′O methylation of rRNA have been reported in patients with DC. USB1 is an outlier, in that it is involved in the processing of the U6 spliceosomal RNA. Dashed arrows link the different proteins to the specific functions in which they are involved. Some of the DC disease genes (such as *DCLRE1B* and *TYMS::ENOSF1)* are not included in this Figure as they do not easily fit into the cartoon scheme.

also been identified in some patients with idiopathic pulmonary fibrosis, liver disease and leukaemia.

In 2008, variants in a component (TIN2) of the shelterin complex were identified in one subtype of AD-DC. The shelterin complex (comprised of six proteins) has at least three effects on telomeres. It determines the structure of the telomeric terminus, it has been implicated in the generation of t-loops and it controls the synthesis of telomeric DNA by telomerase. The composition and protein interaction of the components of shelterin complex appears to be highly ordered, with TIN2 playing a pivotal role. In a subset of patients with DC, HH, AA and Revesz syndrome, heterozygous TIN2 variants have been identified. This discovery extends the range of the DC spectrum of diseases even further. Revesz syndrome is characterised by bilateral exudative retinopathy, BMF, nail dystrophy, fine hair, cerebellar hypoplasia and growth restriction. Patients with TIN2 variants tend to have severe disease and this is associated with very short telomeres. Interestingly, nearly all the patients have *de novo* TIN2 variants, which give rise to a different mechanism causing the disease. In patients with heterozygous *TERC* and *TERT* variants, studies have shown that the phenomenon of genetic anticipation is frequently involved; a parent of an affected child has the same telomerase variant, but usually no overt signs of disease. However, in a child with the same heterozygous telomerase variant the disease manifests itself at a much younger age and is usually more severe.

In recent years heterozygous variants in other genes have been identified that are associated with AD DC or DC-like phenotypes. This includes heterozygous variants in *ACD*, *RTEL1*, *PARN, NAF1*, *ZCCHC8*, *NPM1, MDM4, POT1* and *RPA1*). All these patients usually have short telomeres except those with *NPM1* variants. It is also notable that there is considerable heterogeneity in the age of onset and the number of clinical features seen in the different genetic subtypes.

Autosomal recessive DC

Since 2007 progress has been made in the genetic basis of AR-DC. A large linkage study of 16 consanguineous families comprising 25 affected individuals did not identify a single common locus, suggesting there is genetic heterogeneity within AR-DC. Since this observation, variants in 11 genes have been identified as causing AR-DC. The first AR-DC gene to be identified was *NOP10*. The homozygous *NOP10* variant identified in a large family affected a highly conserved residue. As a result of this variant, all the affected individuals had reduced telomere length and reduced TERC levels. In a subset of AR-DC biallelic variants have been identified in *TERT*. These variants give very different profiles regarding telomerase activity and telomere length, with both being greatly reduced compared with heterozygous *TERT* variants. Biallelic variants in *NHP2* have been identified in a third subset of AR-DC patients. Again, telomere lengths and TERC levels are reduced in patients compared to normal controls. Both NOP10 and NHP2 are components of H/ACA ribonucleoprotein complex (H/ACA RNP). This complex is comprised of a RNA molecule and four proteins, dyskerin, GAR1 as well as NOP10 and NHP2. These four proteins are highly conserved and have been shown to be involved in ribosome biogenesis, pre mRNA splicing and telomere maintenance. Variants have been identified in all components of this H/ACA RNP complex in patients with DC except for GAR1.

In 2011, biallelic variants in *TCAB1* were identified in two patients with AR-DC. TCAB1 is a telomerase holoenzyme protein that facilitates trafficking of telomerase to Cajal bodies, the nuclear sites of nucleoprotein complex modification and assembly. Compound heterozygous variants in TCAB1 disrupt telomere localisation to Cajal bodies, resulting in misdirection of telomerase RNA to nucleoli. This in turn prevents telomerase from elongating telomeres, thereby resulting in short telomeres.

In another subgroup of AR-DC, biallelic variants have been found in *USB1*. It is noteworthy that variants in this gene have unified this subgroup of DC with patients classified as having poikiloderma with neutropenia and Rothmund–Thomson syndrome. This subgroup of AR-DC patients appear to have normal-length telomeres and therefore represents a biologically different subtype.

In 2012, biallelic variants in *CTC1*, encoding conserved telomere maintenance component 1, were identified in a rare subgroup of DC patients. As *CTC1* variants were initially identified in the pleotropic syndrome, Coats plus (characterised by retinopathy, intracranial calcifications and cysts, osteopenia and gastrointestinal abnormalities), this observation expands further the complexity of phenotypes associated with the 'telomeropathies'.

In 2013, biallelic variants in *RTEL1* (regulator of telomere length 1) were identified in some patients with severe DC (close to HH) and these patients have very short telomeres as well as qualitative defects in telomeres. RTEL1 is a helicase with an important role in homologous recombination and telomere maintenance (dismantles telomere t-loops during DNA replication).

More recently, biallelic variants in *PARN* (poly(A)-specific ribonuclease, 2015), *ACD* (adrenocortical dysplasia, 2018), *DCLRE1B* (DNA Cross-Link Repair 1B, 2022) and *TYMS-ENOSF1* (Thymidylate Synthetase and Enolase Superfamily Member 1, 2022) have been identified in patients with DC or DC-like phenotypes, further highlighting the genetic heterogeneity of AR-DC. These patients also have very short/abnormal telomeres.

Treatment

BMF is the main cause of mortality in DC. Use of the anabolic steroids (oxymetholone and danazol) can produce improvement in the haemopoietic function. Approximately two-thirds

of patients with DC will respond to oxymetholone; in some cases, the response can last several years and involve all lineages. Patients with DC can respond to a dose as low as 0.25 mg oxymetholone/kg/day and this can be increased, if necessary, to 2–5 mg/kg/day. It is important to monitor for side-effects (e.g. liver toxicity). It is possible to maintain reasonable blood counts by this approach in many patients. More recently danazol has become to be recognised as a very useful drug in DC patients and is now used as a first-line drug.

The only long-term cure for the haemopoietic abnormalities is allogeneic HSCT. There is significant mortality associated with BM transplants for DC patients than with other BMF syndromes. One of the main reasons for this is the high level of pulmonary/vascular complications that present in these patients, probably as a result of the underlying telomere defect. The conditioning regimen appears to have an impact on patient survival. The standard myeloablative conditioning regimes are associated with frequent and severe adverse effects, such as pulmonary complications and veno-occlusive disease. Recently, the adoption of non-myeloablative fludarabine-based protocols has allowed for successful engraftment in some patients with fewer complications and lower toxicity. The long-term survival, however, is unknown at present, but the initial response is encouraging as a more effective treatment for DC.

New agents (e.g. PAPD5 inhibitors) that can correct the telomere defect *in vitro* represent potentially new treatments for the future.

Shwachman–Diamond syndrome

Clinical features

Shwachman and Bodian and their colleagues first reported this disease independently in 1964. SDS is now recognised as a genetic disorder characterised by exocrine pancreatic insufficiency (100%), BMF (100%) and other somatic abnormalities (particularly involving the skeletal system). Signs of pancreatic insufficiency (malabsorption, failure to thrive) are apparent early in infancy (NB: pancreatic function can improve in a subset of SDS patients). Other somatic abnormalities include short stature (~70%), protuberant abdomen and an ichthyotic skin rash (~60%). Metaphyseal dysostosis is seen on radiographs in about 75% of patients. Other abnormalities include hepatomegaly, rib/thoracic cage abnormalities, hypertelorism, syndactyly, cleft palate, dental dysplasia, ptosis and skin pigmentation.

The spectrum of haematological abnormalities includes neutropenia (~60%), other cytopenias (~20% have pancytopenia), MDS and AML (~25%). The age at which leukaemia develops varies widely, from 1 to 43 years. AML is the commonest category and there is an unexplained preponderance of cases of leukaemia in males.

Exocrine pancreatic insufficiency and haematological abnormalities are also seen in Pearson syndrome (PS), and this is, therefore, an important differential diagnosis. In PS, the anaemia is usually more prominent than neutropenia and the marrow usually shows ringed sideroblasts along with vacuolation of myeloid and erythroid precursors. In addition, acidosis, abnormalities of liver function and mitochondrial DNA rearrangements are seen in PS. PS has a worse prognosis than SDS, with many patients dying before the age of 5 years from liver or BMF. Other differential diagnoses to be excluded are cartilage hair syndrome and cystic fibrosis.

Cell and molecular biology

The SDS gene (*SBDS*) on 7q11.21 was identified in 2003. The majority (>90%) of SDS patients have biallelic variants in this gene (Table 30.7). Data from yeast and human studies suggest that the SBDS protein has an important role in the maturation of the 60S ribosomal subunit (Figure 30.6). These observations suggest that SDS is principally a disorder of ribosome biogenesis. Recently, it has been observed that biallelic variants in *EFL1* and *DNAJC21,* and heterozygous variants in *SRP54* can produce SDS-like disease. Like *SBDS*, *EFL1* and *DNAJC21* also have a role in ribosome biogenesis.

Treatment

The malabsorption in SDS responds to treatment with oral pancreatic enzymes. For those with neutropenia, G-CSF may produce an improvement in the neutrophil count. Some patients

Table 30.7 Genetic subtypes of Shwachman–Diamond syndrome.

SDS subtype	Approximate percentage of patients	Chromosome location	Protein product	Exons
Classical Autosomal recessive	>90	7q11.21	SBDS	5
SDS-like Autosomal recessive	<2	5p13.2	DNAJC21	14
	<2	15q25.2	EFL1	22
Autosomal dominant	<2	14q13.2	SRP54	17

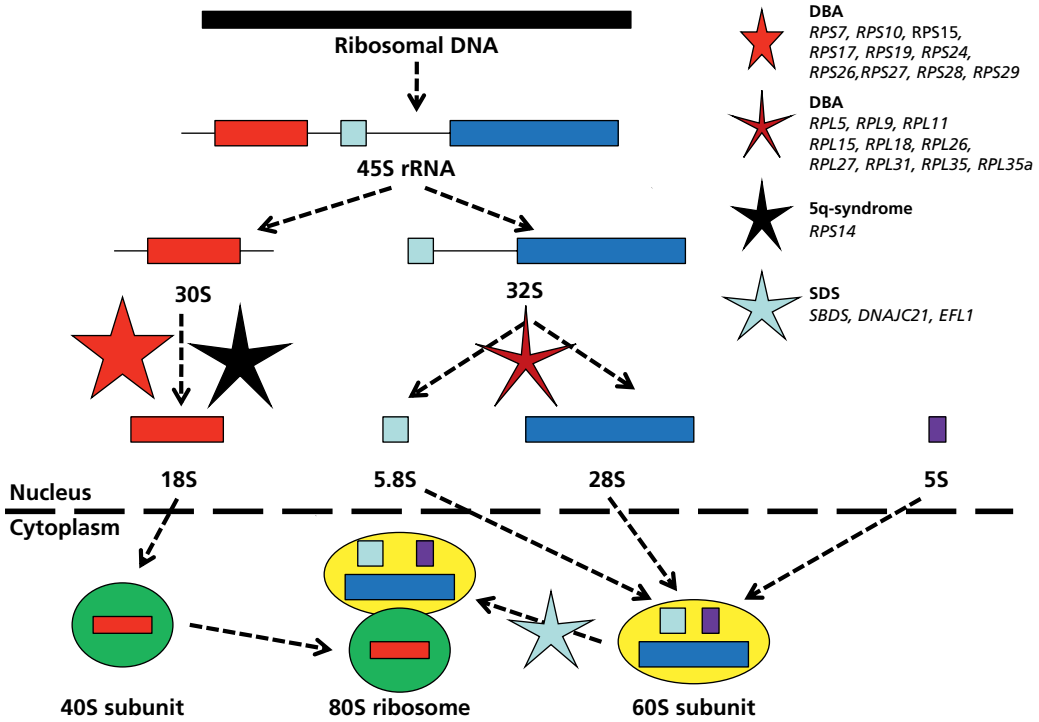


Figure 30.6 Schematic diagram showing scheme of ribosomal (r) RNA processing in human cells and the points at which this is possibly disrupted in the different BMF syndromes. The rRNAs are transcribed by RNA polymerase I as a single precursor transcript (45S rRNA). The 45S rRNA is then processed to 18S, 5.8S and 28S rRNAs. The 18S is a component of the 40S ribosomal subunit. The 5.8S and 28S together with 5S (synthesised independently) are components of the 60S ribosomal subunit. The 40S and 60S subunits are assembled to form the 80S ribosome. The processing steps affected in DBA (heterozygous variants in *RPS7*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPS27*, *RPS28*, *RPS29*, *RPL5*, *RPL9*, *RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, RPL35* and *RPL35a*), 5q– syndrome (haploinsufficiency of *RPS14*) and SDS (biallelic variants in *SBDS, DNAJC21* and *EFL1*) are indicated by the different coloured stars.

with anaemia and/or thrombocytopenia may achieve haematological responses with oxymetholone treatment. As in other types of BMF, supportive treatment with blood products and antibiotics is very important.

The development of leukaemia, often with features of myelodysplasia and *TP53* mutations, usually has a poor prognosis. SDS patients with leukaemia treated with conventional courses of chemotherapy usually fail to regenerate normal haemopoiesis. As this is a constitutional disorder, all somatic cells, including haemopoietic stem cells, are abnormal. In addition, the haemopoietic stem cells may have accumulated secondary abnormalities, as suggested by complex karyotypes (especially involving chromosome 7) often observed in the BM from these patients. Therefore, for those who develop leukaemia, the only approach likely to be successful is allogeneic HSCT, using conditioning regimens that include fludarabine. It is noteworthy that non-haematological malignancies have not been observed in SDS patients.

Diamond–Blackfan anaemia (DBA)

Clinical features

Red cell aplasia was first reported in 1936 by Josephs. In 1938, Diamond reported on four children with hypoplastic anaemia and this has now come to be recognised as DBA or congenital pure red cell aplasia. DBA usually presents in early infancy, with features of anaemia such as pallor or failure to thrive. The hallmark of classical DBA is a selective decrease in erythroid precursors and normochromic macrocytic anaemia associated with a variable number of somatic abnormalities such as craniofacial, thumb, cardiac and urogenital malformations. Hitherto the diagnostic criteria for DBA have comprised: (i) normochromic, usually macrocytic, but occasionally normocytic anaemia developing in early childhood; (ii) reticulocytopenia; (iii) normocellular BM with selective deficiency of erythroid precursors (erythroblasts <5%); (iv) normal or slightly decreased leucocyte

counts and (v) normal or often increased platelet counts. More recently, elevated erythrocyte deaminase activity, macrocytosis and elevated fetal haemoglobin levels have been added to the list of supportive features of DBA. It has also been recognised that in a subset of cases, the presentation may be in adulthood.

There is considerable heterogeneity in the associated somatic abnormalities and response to therapy. Analysis of cases recruited to the DBA Registry of North America (DBAR) has shown that the annual incidence of DBA is about 5 per million live births. The median age at presentation was 8 weeks and 93% of patients presented in the first year of life. In total, 79% were initially responsive to steroids, 17% were non-responsive and 4% were never treated with steroids; 31% of patients were receiving transfusions at analysis. The actuarial survival rates older than 40 years were 100% for those in sustained remission, 87% for steroid-maintained patients and 57% for transfusion-dependent patients. Of the 36 deaths reported, 25 were treatment-related: 5 from infections, 5 from complications of iron overload, 1 related to vascular access and 14 from transplant-related complications.

In the DBAR, 8.8% of families had more than one affected individual. Most of the familial cases displayed AD inheritance. Somatic anomalies, excluding short stature, were found in 47% of patients. Of these, 50% were craniofacial (high-arched palate, cleft lip, hypertelorism and flat nasal bridge), 38% were upper limb and hand (flat thenar eminence, triphalangeal thumb), 39% genitourinary and 30% cardiac. Height was below the third centile for age in about 30%.

MDS and AML have been reported in a few patients with DBA. There are also cases that have evolved into AA; neutropenia and thrombocytopenia are relatively common after the first decade. Giri and colleagues reported on moderate to severe BM hypocellularity in 21 of 28 (75%) patients with steroid-refractory DBA; marrow hypoplasia correlated with the development of neutropenia (9/21, 43%) and/or thrombocytopenia (6/21, 29%). Furthermore, using *in vitro* long-term culture-initiating cell assay, they provided evidence for a trilineage haemopoietic defect in patients with refractory DBA. Thus, although DBA has been regarded classically as a pure red cell aplasia, a more global haemopoietic defect is likely to be present, and this may be seen more frequently in the future as patients survive longer due to improved medical care.

Cell and molecular biology

A number of different defects of *in vitro* erythroid progenitor proliferation, differentiation and cytokine responsiveness have been reported. The establishment of DBA registries and advances in genetics have led to genetic categorisation of many DBA cases. The first gene to be identified was *RPS19* (1999). Subsequently, heterozygous variants in other genes encoding proteins of the small (RPS7, RPS10, RPS15, RPS17, RPS24, RPS26, RPS27, RPS28 and RPS29) and large (RPL5, RPL9, RPL11, RPL15, RPL18, RPL26, RPL27, RPL31 and RPL35A) ribosomal subunits have also been reported (Table 30.8); collectively the genetic basis of approximately 75% of DBA patients can now be substantiated at the genetic level. These findings suggest that the primary defect in DBA is defective ribosome biogenesis, which then leads to other biological defects, including increased apoptosis and upregulation of p53. It will now be important to establish how precisely variants in these ribosomal protein genes lead to altered cell growth (and anaemia), developmental anomalies and increased susceptibility to cancer. A subgroup of patients with DBA/DBA-like disease have been found to be associated with variants in *GATA1*, *CECR1*, *TSR2* and *EPO*.

Review of clinical and genetic data has shown that patients with *RPL5* variants tend to have multiple physical abnormalities, including craniofacial, thumb and heart anomalies, whereas isolated thumb malformations are predominantly seen in patients with heterozygous *RPL11* variants. Some genotype–phenotype correlations are therefore beginning to emerge.

It is noteworthy that recent advances have provided a very interesting connection between DBA, SDS, MDS and defective ribosome biogenesis (see Figure 30.6). Specifically: (i) DBA (as highlighted here) is caused by variants in genes important in the biogenesis of the small and large ribosomal units; (ii) the SDS genes (see above) are required for the maturation of the large ribosomal unit; and (iii) the 5q – syndrome (a recognised subtype of acquired myelodysplasia) is associated with haploinsufficiency of the gene encoding the ribosomal protein RPS14. These advances highlight the importance of studies on rare syndromes, as often they can provide significant insights into the pathology of more common diseases.

Treatment

The first line of treatment for DBA remains corticosteroids. Once a maximal haemoglobin response has been achieved, the dose of prednisolone should be tapered slowly until the patient is on the lowest dose possible on an alternate-day regimen. For those patients who fail to respond or become refractory to steroids, blood transfusion is the mainstay of treatment. The major complication from transfusions is iron overload, and chelation of iron (avoiding deferiprone) should therefore be commenced as soon as patients have increased iron stores (Chapter 12). Splenectomy may be indicated in the event of an increased transfusion requirement secondary to hypersplenism. For patients who are transfusion-dependent and who have a HLA-compatible donor, HSCT may be appropriate.

Congenital dyserythropoietic anaemia

CDA comprises a heterogeneous group of disorders of erythropoiesis characterised by anaemia, ineffective erythropoiesis and frequently morphological evidence of dyserythropoiesis.

Table 30.8 Genetic subtypes of Diamond–Blackfan anaemia.

DBA subtype	Approximate percentage of patients	Chromosome location	Protein product	Exons
Autosomal dominant	25	19q13.2	RPS19	6
	10–20	Various*		
	7	1p22.1	RPL5	8
	7	12q13.2	RPS26	4
	5	1p36.11	RPL11	6
	3	3q29	RPL35A	5
	3	6q21.31	RPS10	6
	2.4	10q22.3	RPS24	9
	1	15q25.2	RPS17	6
	<1	3p24.2	RPL15	5
	<1	2p25.3	RPS7	7
	<1	19p13.2	RPS28	4
	<1	14q21.3	RPS29	5
	<1	17p13.1	RPL26	4
	<1	19p13.3	RPS15	4
	<1	1q21.3	RPS27	4
	<1	4p14	RPL9	8
	<1	19q13.33	RPL18	7
	<1	17q21.31	RPL27	6
	<1	2q11.2	RPL31	5
X-linked recessive	<1	Xp11.23	GATA1	6
	<1	Xp11.22	TSR2	5
Uncharacterised	~25	?	?	?

* This refers to large deletions in different DBA genes. Variants in *EPO* and *CECR1/DADA2* can also produce DBA-like disease.

The first description of these disorders was in 1966 by Crookston and colleagues. In 1968 Wendt and Heimpel classified CDA into three types (I, II and III) (Table 30.9). Over the years many additional subtypes (IV, V, VI and VII) have been added to the list, often based on case reports.

CDA type I

The majority of patients present with splenomegaly and mild to moderate anaemia (~66–116 g/L); approximately 70% have macrocytosis. In some cases, non-haematological features (e.g. skeletal abnormalities and abnormal skin pigmentation) have been observed. Ineffective erythropoiesis is evidenced by morphological abnormalities in the peripheral blood (anisocytosis) and in the marrow (megaloblastic erythroid precursors, internuclear chromatin bridging, binuclearity affecting 3–7% of erythroblasts; Figure 30.7a), as well as by increased markers of haemolysis (elevated lactate dehydrogenase and bilirubin). The defining ultrastructural feature is a spongy ('Swiss cheese') appearance of the heterochromatin in the majority of erythroblasts on electron microscopy (Figure 30.7b).

Recognised to be AR, the first gene responsible for CDA type I (*CDAN1,* encoding codanin) was identified in 2002. Recently a second gene, *CDIN1*, was found to be responsible for some cases of CDA type I.

CDA type II

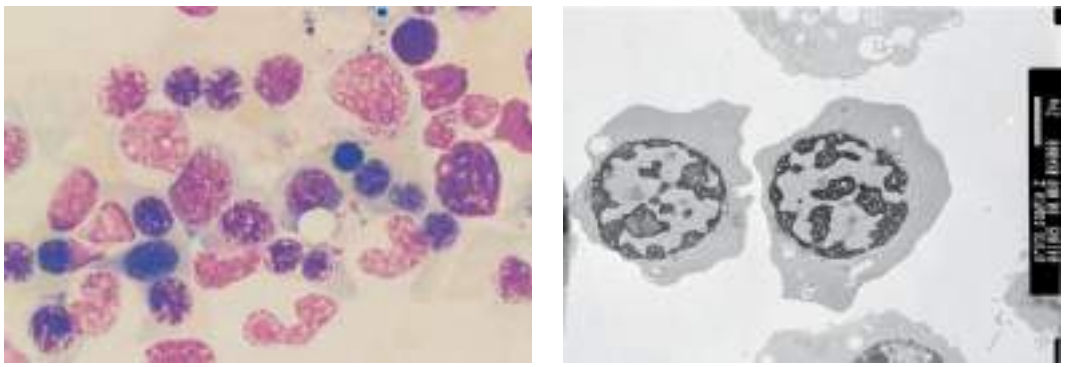
This is the most common subtype of CDA and was initially described as hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS) in 1969. It is inherited as an AR trait. The anaemia is variable (Hb 80–110 g/L). Approximately 10% of cases require regular transfusions and some cases present with anaemia at birth. The clinical presentations include a variable degree of jaundice, hepatomegaly, splenomegaly and cirrhosis. Mental restriction has been reported in some cases.

Peripheral blood morphology shows moderate to marked red cell anisocytosis. BM features include normoblastic erythroid hyperplasia with usually more than 10% binucleate erythroblasts (Figure 30.8a). At the electron microscope level, the erythroid cells have a characteristic peripheral arrangement of the endoplasmic reticulum giving the appearance of a 'double membrane'

Table 30.9 Characteristics of common subtypes of congenital dyserythropoietic anaemia.

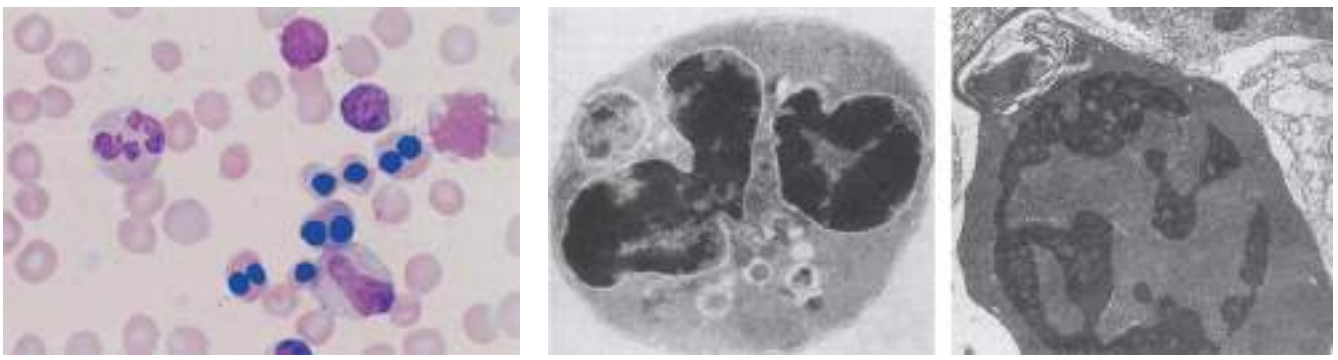
Feature	Type I	Type II	Type III
Inheritance	AR	AR	AD
Erythrocytes	Macrocytic	Normocytic	Macrocytic
Erythroblasts			
Light microscopy	Megaloblastic, internuclear chromatin bridges	Normoblastic, binuclearity	Megaloblastic, up to 12 nuclei/cell
Electron microscopy	'Swiss-cheese' appearance	Peripheral double membranes	Non-specific
Serology			
Ham test	Negative	Usually positive	Negative
Anti-i-agglutinabiliy	Normal/strong	Strong	Normal/strong
SDS-PAGE	Normal	Band 3 thinner and migrates faster than normal	Band 3 migrates faster than normal
Genes identified	*CDAN1* (15q15.2), *CDIN1* (15q14)	*SEC23B* (20p11.23)	*KIF23* (15q23)

(*Source:* Wickramasinghe SN, Wood WG (2005) Advances in the understanding of congenital dyserythropoietic anaemia. *Br. J. Haematol.* **131**: 431–46. Reproduced and modified with permission of John Wiley & Sons.)



(a) (b)

Figure 30.7 (a) CDA type I. BM aspirate showing internuclear bridging in normoblasts. (b) CDA type I. Electron micrograph of erythroblasts showing a spongy ('Swiss cheese') appearance of the heterochromatin (Source: David Fergusion and Noemi Roy. Reproduced with permission.)



(a) (b)

Figure 30.8 (a) CDA type II (HEMPAS). BM aspirate showing typical multinuclearity. (b) CDA type II. Electron micrographs of erythroblast showing peripheral arrangement of endoplasmic reticulum with 'double membrane' appearance.

(Figure 30.8b). Red cells from patients with CDA type II are haemolysed by some acidified sera, but not by the patient's own serum. In 2009 the gene encoding the secretory COPII component SEC23B was shown to be responsible for CDAII.

CDA type III

This subtype is rare. In one of the largest (Swedish) families investigated, the disease was characterised by giant multinucleated erythroblasts in the marrow (Figure 30.9). There appears to be an increased prevalence of lymphoproliferative disorders in CDA type III. CDA III exhibits AD transmission and is caused by variants in *KIF23*. *KIF23* encodes mitotic kinesin-like protein 1, which plays a critical role in cytokinesis during cell division.

Treatment

Those with mild anaemia require no major intervention. Folate supplementation should be given to prevent folate deficiency.

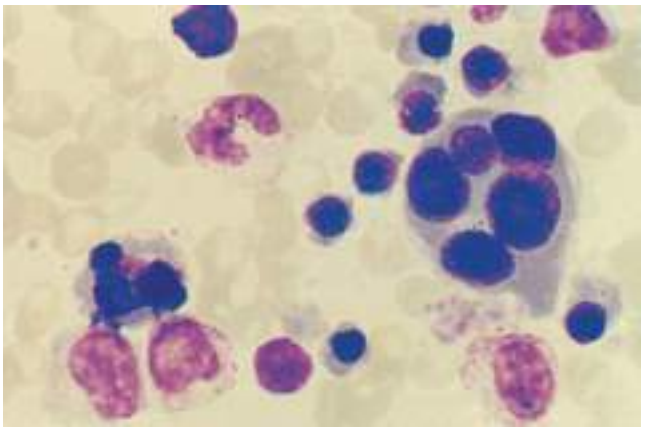


Figure 30.9 CDA type III. Giant multinucleated erythroblast from the marrow.

If regular transfusions are necessary, early attention to iron chelation is essential. Splenectomy may be of benefit in some patients (CDA type II) and there are rare case reports of successful HSCT. In CDA type I there are also case reports of improvement in the haemoglobin after treatment with interferon-α. The mechanism of this therapeutic benefit remains unclear.

Congenital and cyclical neutropenias

Congenital neutropenia is a heterogeneous disorder (Table 30.10). It includes Kostmann syndrome, which was first described in 1954. Although the original description by Kostmann was of an AR disorder, other congenital neutropenia subtypes (both sporadic and AD) have subsequently been included in this category. The neutropenia is usually recognised at birth and the neutrophil count is often below 0.2×10^9/L. The haemoglobin and platelet count are usually normal and the BM shows maturation arrest of myelopoiesis at the level of the promyelocyte/myelocyte (with abundant promyelocytes, but with selective reduction in myelocytes, metamyelocytes and neutrophils).

The neutropenias are associated with severe infections and early death. No patient has developed AA, but myeloid leukaemias (~25% by 25 years) can occur. The availability of G-CSF has revolutionised the outcome for these children. These patients can progress to MDS and leukaemia, usually with acquisition of secondary mutations including in the G-CSF receptor. The precise contribution of G-CSF therapy to the development of G-CSF receptor mutations remains unclear. For patients who become refractory to G-CSF or who develop leukaemia, HSCT may be appropriate and curative.

Cyclical neutropenia is characterised by a neutrophil count that usually reaches a nadir with a 21-day periodicity. Around the nadir, patients may develop fever and mouth ulcers. Cyclical neutropenia is usually AD. Linkage analysis in affected families resulted in the localisation of the disease gene to

Table 30.10 Genetic subtypes of neutropenia.

Subtype	Approximate percentage of patients	Chromosome location	Gene/protein product	Exons
Autosomal dominant	50–60	19p13.3	*ELANE*	6
	<2	1p22.1	*GFI1*	9
Autosomal recessive	10–15	1q21.3	*HAX1*	7
	~5	17q21.31	*G6PC3*	8
	Rare	1q21.2	*VPS45*	18
	Rare	1p34.3	*CSF3R*	19
	Rare	3p25.3	*JAG1*	2

NB: Some patients initially presenting as isolated neutropenia may turn out to be cryptic presentations of other syndromes, such as Wiskott–Aldrich syndrome.

19p13.3. Subsequent studies identified variants in the gene (*ELANE*) encoding neutrophil elastase. An extraordinary twist to the story was the identification of *ELANE* variants in many patients with congenital neutropenia as well. In cyclical neutropenia, the variants are usually clustered around the active site of the molecule, whereas the opposite face of the molecule tends to be mutated in congenital neutropenia. Neutrophil elastase is a serine protease that is synthesised predominantly at the promyelocytic stage and can be expected to be important in neutrophil development. Recent studies suggest that *ELANE* variants lead to accumulation of the non-functional protein, which in turn triggers an unfolded protein response leading to maturational arrest. The precise mechanism leading to maturation arrest of promyelocytes remains unclear. The original family described by Kostmann had AR severe congenital neutropenia and has been shown to be associated with biallelic variants in *HAX1*, predicted to lead to defects in cell death. Biallelic variants in *HAX1* account for approximately 10% of congenital neutropenia. The HAX1 protein is a critical regulator of the mitochondrial membrane potential and cellular viability. While there are data that suggest that HAX1 is important in controlling apoptosis, it is unclear why premature death of neutrophils is consistently associated with HAX1 deficiency. Variants in other genes (*GFI1, G6PC3* and *VPS45*) are also associated with SCN.

Thrombocytopenia with absent radii (TAR)

TAR is an AR disorder characterised by hypomegakaryocytic thrombocytopenia and bilateral radial aplasia. Babies with TAR often have haemorrhagic manifestations at birth, when the diagnosis is usually made because of the characteristic physical appearance combined with thrombocytopenia. Additional skeletal abnormalities (absent ulnae, absent humeri and clinodactyly) and other somatic abnormalities (microcephaly, hypertelorism, strabismus and heart defects) may be seen in some patients.

The platelet count is usually below 50×10^9/L. The leucocyte count can be normal or raised, sometimes up to 100×10^9/L ('leukaemoid reaction'). BM cellularity is normal and myeloid and erythroid lineages are normal or increased. Megakaryocytes are absent or decreased. Most patients bleed in infancy and then improve after the first year. The mainstay of management is prophylactic and therapeutic use of platelet transfusions. Patients with TAR have a very good prognosis after infancy. There have been no reports of AA or leukaemia.

In TAR patients, thrombopoietin levels are usually elevated and thrombopoietin receptor expression on the surface of TAR platelets is normal. Therefore, defective megakaryocytopoiesis/thrombocytopoiesis does not appear to be caused by a defect in thrombopoietin production. Recently it has been determined that compound inheritance of low-frequency regulatory SNP and a rare null variant in *RBM8A* (which encodes a subunit of the exon-junction complex) causes TAR.

Congenital amegakaryocytic thrombocytopenia (CAMT)

CAMT is a rare disorder that usually presents in infancy and is characterised by isolated thrombocytopenia and reduction/absence of megakaryocytes in the BM, usually with no somatic abnormalities. It is genetically heterogeneous with AR and X-linked subtypes. Approximately 50% of patients will develop AA, usually by the age of 5 years. For patients with severe thrombocytopenia or AA, the treatment of choice is HSCT, if a compatible donor is available.

In a subgroup of patients with CAMT, biallelic variants in the gene encoding the thrombopoietin receptor (*MPL*) have been identified (Table 30.11). As patients with *MPL* variants can also have abnormalities in the leucocyte count and haemoglobin

Table 30.11 Genetic subtypes of syndromic thrombocytopenias.

Subtype	Approximate percentage of patients	Chromosome location	Gene/locus	Exons
CAMT Autosomal recessive	Majority	1p34	*MPL*	11
TAR Autosomal recessive	Majority	1q21.1	*RBM8A*	6
Radio-ulnar synostosis Autosomal dominant	Major subset	7p15.2	*HOXA11*	2
	Major subset	3q26.2	*MECOM**	23

TAR = thrombocytopenia with absent radii.
* *MECOM* (MDS1 and EVI1 complex locus) variants can be associated with variable haematological features ranging from isolated thrombocytopenia to global BM failure, MDS and leukaemia.

level and central nervous system (CNS) abnormalities (e.g. cerebral and cerebellar hypoplasia), this highlights the important role of the thrombopoietin receptor in haemopoiesis in general and in CNS development. It also substantiates the genetic heterogeneity of CAMT.

New subtypes of inherited BM failure and overlapping syndromes

There are cases of BM failure where they are familial and/or have one or more extra-haemopoietic/somatic abnormality but which do not fit into the entities listed above. The availability of next-generation sequencing has made it possible to elucidate the genetic basis of some of these. Examples of these new entities include those associated with germline variants (Table 30.12) in *TPO*, *ERCC6L2*, *MYSM1*, *DUT*, *EXOC3L2, TP53* and *SP1*. The number of cases reported in each subtype is variable.

It is also notable that there are entities that were initially characterised in patients with myelodysplasia/leukaemia or other syndromic diseases but which can also present with peripheral cytopenias. This includes GATA2 deficiency and SAMD9/SAMD9L-related disease. In addition to MDS and leukaemia, these patients can have a variable number of extra-haemopoietic abnormalities. Germline variants in these genes are particularly prevalent in paediatric patients with MDS associated with monosomy 7. *GATA2* and *SAMD9/SAMD9L* are also included in the category of familial MDS/AML genes. Other genes in this familial MDS/AML category include: *RUNX1*, *CEBPA*, *TERC*, *TERT*, *SRP72, AKNRD26, ETV6*, *DDX41*, *RTEL1*, *PAX5*, *TP53*, *ACD*, *MECOM*, *HLTF*, *XPC and DHX34* (Table 30.12). This highlights the overlapping nature of the haematological (BMF, MDS and AML) and extra-haematological phenotypes produced by germline variants in these genes. It is likely that additional new entities of familial BM failure/MDS will be characterised in the future.

Table 30.12 New BMF and overlapping syndromes.

Subtype	Chromosome location	Gene product	Exons
Recently recognised BMF subtypes			
Autosomal recessive	9q22.32	*ERCC6L2*	27
	3q27.1	*TPO/THPO*	7
	1p32.1	*MYSM1*	23
	15q21.1	*DUT*	9
	19q13.32	*EXOC3L2*	10
	17p13.1	*TP53*	12
Autosomal dominant	7q21.3	*SAMD9**	3
	7q21.2	*SAMD9L**	6
	12q13.13	*SP1*	7
Familial myelodysplasia and leukaemia			
Autosomal dominant			
	21q22.12	*RUNX1*	13
	19q13.11	*CEBPA*	1
	3q26.2	*TERC**	1
	5p15.33	*TERT**	16
	3q21.3	*GATA2**	8
	4q12	*SRP72*	20
	10p12.1	*ANKRD26*	46
	16q22.1	*ACD/TPP1*	12
	12p13.2	*ETV6*	14
	5q35.3	*DDX41*	17
	20q13.33	*RTEL1*	35
	9p13.2	*PAX5*	11
	7q21.3	*SAMD9**	3
	7q21.2	*SAMD9L**	6
	3q26.2	*MECOM**	23
	17p13.1	*TP53*	12
	12q13.2	*ERBB3*	28
	19q13.32	*DHX34*	21
Autosomal recessive			
	3q21.3	*MBD4*	8
	3q24	*HLTF*	25
	3p25.1	*XPC/XPCC*	18

* Variants in these genes can produce very diverse haematological features including AA, MDS and leukaemia. They can also produce a variable range of extra-haematopoietic abnormalities. For example: GATA2 deficiency can be associated with pulmonary alveolar proteinosis and primary lymphedema; SAMD9 disease with adrenal insufficiency, intrauterine growth restriction and genital abnormalities; SAMD9L disease with neurologic/cerebellar, ophthalmic abnormalities.

Conclusion

Since the identification of the first FA gene (*FANCC*) in 1992, there have been significant advances in our understanding of FA, DC and other BMF syndromes. This has facilitated diagnosis, as highlighted in Table 30.13. It can be anticipated that further studies of the pathophysiology of these disorders are likely to lead to a better understanding of normal haemopoiesis and how this becomes defective in many patients presenting with the more common forms of AA and MDS. Indeed, studies have already established a link between DC and AA and, in turn, to defective telomere maintenance. Equally, a link between DBA, SDS and MDS and, in turn, defective ribosome biogenesis has been recognised. These advances also suggest that new treatment strategies, based on targeting the primary defect in each syndrome, are likely to emerge in the future.

Table 30.13 Laboratory tests useful in the investigation of patients with BMF.

Test	Diagnostic value
Peripheral blood	
Fetal haemoglobin	High level suggestive of generalised BMF
DEB/MMC-induced chromosomal breakage	Increased in FA
Genetic analysis using next-generation sequencing	
FANCA–FANCW	Mutated in FA
DKC1, TERC, TERT, NOP10, NHP2, TINF2, TCAB1 USB1, CTC1, RTEL1, ACD, PARN, NAF1, ZCCHC8, NPM1, MDM4 POT1, RPA1, DCLRE1B and TYMS-ENOSF1	Mutated in DC and related disorders
SBDS, DNAJC21, EFL1, SRP54	Mutated in SDS and SDS-like disease
RPS19, RPS7, RPS10, RPS15, RPS17, RPS24, RPS26, RPS27, RPS28, RPS29, RPL5, RPL9, RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, RPL35A, GATA1, TSR2, EPO	Mutated in DBA and DBA-like disease
CDAN1, CDIN1, SEC23B, KIF23, KLF1	Mutated in CDA and CDA-like disease
ELANE, HAX1, GFI1, G6PC3, VPS45, JAG1	Mutated in congenital and cyclic neutropenia
MPL, RBM8A, HOXA11 and MECOM	Mutated in CAMT and other syndromic thrombocytopenias
TPO, ERCC6L2, MYSM1, DUT, EXOC3L2, TP53 and SP1	Mutated in newly recognised BMF syndromes
RUNX1, CEBPA, GATA2, SAMD9, SAMD9L, TERC, TERT, SRP72, AKNRD26, ETV6, DDX41, RTEL1, PAX5, TP53, ACD, MECOM, HLTF, XPC and DHX34	Mutated in overlapping BMF/MDS/leukaemia syndromes
Mitochondrial DNA analysis	Deletions seen in PS
X-chromosome inactivation patterns	Skewed in carriers of X-linked DC
Ham test (CD59 analysis)	Abnormal in paroxysmal nocturnal haemoglobinuria
Telomere length	Short in AA, very short in DC
Constitutional karyotype	Abnormality suggestive of constitutional AA
Other investigations	
To identify somatic abnormalities	Presence of somatic/extra-haematopoietic abnormalities in association with AA is suggestive of constitutional/inherited AA
Skeletal survey	
Ultrasound of abdomen	
Pulmonary function tests	
Echocardiogram	
Exocrine pancreatic function	Abnormal in SDS and PS
Neutrophil chemotaxis	Abnormal in SDS
Fibroblast cultures	Abnormalities seen in DC

AA, aplastic anaemia; CAMT, congenital amegakaryocytic thrombocytopenia; CDA, congenital dyserythropoietic anaemia; DBA, Diamond–Blackfan anaemia; DC, dyskeratosis congenita; DEB/MMC, diepoxybutane/mitomycin-C; FA, Fanconi anaemia; PS, Pearson syndrome; SDS, Shwachman–Diamond syndrome.

Acknowledgements

I would like to thank my research colleagues past (Richard Beswick, Shirleny Cardosa, Laura Collopy, Michael Kirwan, Stuart Knight, Anna Marrone, Jasmin Sidhu, David Stevens and Philip Mason) and present (Jenna Alnajar, Jude Fitzgibbon, Upal Hossain, Hemanth Tummala, Amanda Walne and Tom Vulliamy) and all the patients and clinicians on whom research in our laboratory depends. The research has been supported by significant funding from the Wellcome Trust, Blood Cancer UK and the Medical Research Council UK.

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CHAPTER 31

Acquired aplastic anaemia and paroxysmal nocturnal haemoglobinuria

31

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Acquired aplastic anaemia

Characterisation and definition

Aplastic anaemia (AA), a rare bone marrow failure syndrome (BMFS), is defined by pancytopenia with a hypocellular bone marrow in the absence of an abnormal infiltrate and with no increase in reticulin. The term 'aplastic anaemia' encompasses different entities, but here we discuss acquired AA, most often idiopathic, though sometimes a drug or chemical or a virus infection is implicated. 'Inevitable' AA/myelosuppression occurs after treatment with cytotoxic drugs or radiation; it is dose-dependent and recovery is usually predictable and is discussed no further here.

AA is a bone marrow failure disorder and shows considerable overlap with clonal disorders of bone marrow failure, including myelodysplastic syndrome (MDS), acute myeloid leukaemia (AML), paroxysmal nocturnal haemoglobinuria (PNH) and T-large granular lymphocyte leukaemia/lymphoproliferative disorder (T-LGL), and a tendency itself to later evolve to MDS/AML (Figure 31.1). The inherited forms of AA, such as Fanconi anaemia (FA), dyskeratosis congenita (DC) and Shwachmann–Diamond syndrome (SDS), are rarer than acquired AA and are discussed in detail in Chapter 30. However, the importance of excluding an inherited form of AA and the increasing proportion of adults with apparent acquired AA with related inherited genetic lesions is highlighted in this chapter. In AA, there must be at least two of the following: (i) haemoglobin below 100 g/L, (ii) platelet count below 50×10^9/L and (iii) neutrophil count below 1.5×10^9/L. Patients with bilineage or trilineage cytopenias that are less severe than this are not classified as AA. However, they should have their blood counts monitored to determine whether they will develop AA later. The severity of the disease is graded into very severe, severe and nonsevere (or moderate) AA, according to the blood count parameters and bone marrow findings, as summarised in Table 31.1. The assessment of disease severity is important in treatment decisions, prediction of response to immunosuppressive therapy (IST) and is also of prognostic significance.

Epidemiology

Aetiology and incidence

Studies indicate an incidence of AA in the West of 2 per million per year. There is a twofold to threefold higher incidence rate in Asia. In a large prospective study from Thailand, an incidence of 3.9 per million was reported from the metropolitan area of Bangkok compared with 5 per million in the northeast region of Khonkaen. An incidence of 7.4 per million was reported from a prospective study from China, although this may represent an overestimate, as a bone marrow trephine was not required for the diagnosis of AA. The reasons for the differences in incidence are not known but may include environmental and genetic factors.

There is a biphasic age distribution, with peaks at 10–25 years and over 60 years; it is possible that some cases diagnosed in

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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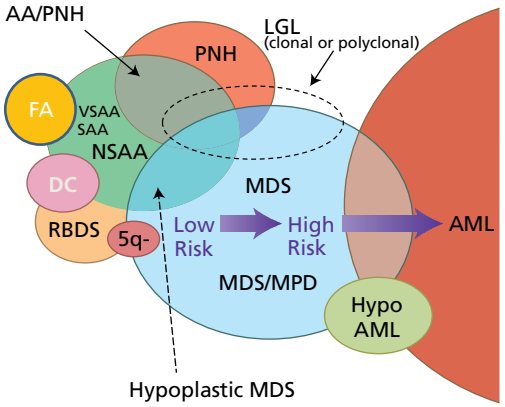


Figure 31.1 Overlapping bone marrow failure disorders. AA, aplastic anaemia; AML, acute myeloid leukaemia; DC, dyskeratosis congenita; FA, Fanconi anaemia; LGL, large granular lymphocytosis; NSAA, non-severe AA; PNH, paroxysmal nocturnal haemoglobinuria; RBDS, ribosomal dysgenesis syndromes; SAA, severe AA; VSAA, very severe AA.

older patients represent hypocellular MDS rather than AA. There is no significant difference in incidence between males and females.

Posthepatitic AA accounts for around 5–10% of cases; invariably such cases are negative for the known hepatitic viruses. Patients, typically young males, present with jaundice and hepatitis symptoms, then, on average 6 weeks later, develop pancytopenia when the liver function has usually improved or normalised. Posthepatitic AA is usually severe, but responds well to IST, and similar oligoclonal expansion of activated cytotoxic T cells is observed as in idiopathic AA, suggesting a common pathogenesis for the hepatitis and bone marrow failure. Rarely, AA may follow EBV-related infectious mononucleosis.

AA occurs in association with other systemic autoimmune disorders, especially eosinophilic fasciitis, but also systemic lupus erythematosus (SLE), Sjögren syndrome and coeliac disease. SLE can also produce pancytopenia with a cellular bone marrow or it may occasionally be associated with marrow fibrosis. Thymoma may be associated with AA, although typically related to pure red cell aplasia (PRCA). Fatal AA is almost invariably the outcome of transfusion-associated graft-versus-host disease (GVHD); it is well-documented clinically and can be reproduced *in vivo* in a mouse model.

The association between AA, other autoimmune disorders and TLGL, clonal proliferation of cytotoxic ($CD8^+$) T cells, has been known for decades and conceptualises the theory of immune-mediated marrow suppression by a dominant T cell clone. Somatic *STAT3* mutations in autoimmune T cells in a proportion (7%) of AA possibly induces a persistently dysregulated autoimmunity, which is amenable to immunosuppression, akin to LGL leukaemia.

AA can rarely occur in pregnancy, although this may be due to chance and other possible causes should always be sought. A retrospective review from Leiden over a 24-year period found a similar frequency of AA in pregnancy compared to that in the general population. However, due to the rarity of AA, this may not exclude a possible association in a larger number of patients. The disease may remit spontaneously after termination, whether spontaneous or therapeutic, and after delivery, but not in all cases. There is a risk of relapse in pregnancy in patients who have previously responded to IST. In contrast, after successful allogeneic haemopoietic stem cell transplantation (HSCT), pregnancy does not trigger relapse.

Pathogenesis and its clinical relevance

The haemopoietic defect in AA

AA is characterised by both quantitative and qualitative defects in the haemopoietic stem and progenitor cell (HSPC) compartment. The primitive long-term culture-initiating cells and more mature haemopoietic progenitors in the bone marrow (colony-forming cells) of all cell lineages are reduced or absent. In addition to the reduced numbers and impaired repopulating ability of bone marrow $CD34^+$ cells, these cells are more apoptotic than normal $CD34^+$ cells, as evidenced by transcriptome analysis. About 10–15% of patients with AA have shortened telomeres, as measured in blood leucocytes (see later).

The bone marrow stromal cell microenvironment functions normally in most patients as assessed by an *in vitro* system, although this does not examine individual cellular components of the stroma and thus defects in particular cells cannot be excluded. The frequencies of fibroblast colonies (CFU-F) are normal in AA, and mesenchymal stem cells showed normal phenotype as defined by $CD34^-$ $CD45^-$ $CD44^+$ $CD29^+$ $CD90^+$ $CD105^+$ $CD106^+$. The differentiation capacity of AA mesenchymal stem cells has not yet been formally evaluated. Cultured AA mesenchymal stem cells support *in vitro* haemopoiesis after addition of normal bone marrow mononuclear cells.

AA is not due to a deficiency of any known haemopoietic growth factor (HGF). Long-term marrow culture studies have shown normal mRNA expression and/or secretion of granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), interferon-gamma (IFN-γ), interleukin (IL)-6, stem cell factor and thrombopoietin (TPO) from stromal cells. Serum levels of most HGFs, including the above and erythropoietin, are markedly elevated. These observations explain the lack of striking effects of HGFs in most AA patients, although recent data suggest a role for synthetic TPO-mimetic eltrombopag in AA, possibly circumventing the inhibitory effect of IFN-gamma by binding to an alternate site on TPO receptor and enhancing HSPC function.

Table 31.1 Laboratory assessment of a newly presenting patient.

1. *Laboratory tests to confirm diagnosis*	
Full blood count	Pancytopenia with reticulocytopenia. Single cytopenia especially thrombocytopenia may precede pancytopenia. Raised MCV common.
Blood film examination	Toxic granulation of neutrophils, anisopoikilocytosis, macrocytosis. Exclude blasts, dysplastic neutrophils and hairy cells.
Liver function tests, vitamin B12 and folate	To detect preceding hepatitis; exclude and correct coexisting vitamin B12 or folate deficiency.
Virology	Post-hepatitic AA occurs in 5–10% cases, serology usually negative for known hepatitis viruses; HIV and parvovirus are very rare causes of AA; check CMV exposure for potential BMT candidates.
Antinuclear antibody and dsDNA	SLE is very rare cause of AA, and other autoimmune syndromes associated with pancytopenia.
Radiology	Chest X-ray to exclude infection; HRCT scan of chest to exclude pulmonary fibrosis if DC or familial BMF suspected. Abdominal ultrasound or CT scan: hepatic fibrosis in DC; splenomegaly or lymphadenothy indicate alternative diagnosis; abnormal or misplaced kidneys may occur in Fanconi anaemia.
BM examination	*Aspirate* fragments easily obtained, hypocellular fragments and trails. Reduced/absence of haemopoietic lineages. Dyserythropiesis common. Absence of dysplasia in granulocytic and megakaryocytic lineages. Lymphocytes, plasma cells, macrophages and mast cells often prominent. Iron stain to exclude ringed sideroblasts. CD34+ immunostain negative. *Trephine*: Good length (at least 1–2 cm) essential. Hypocellular (Figure 31.3). May be patchy cellularity. Absence of blasts. Lymphoid aggregates (reactive) are common. Reticulin stain negative. CD34 immunocytochemistry negative. CD61 useful to exclude dysplastic megakaryocytes.
2. *Tests to detect an associated abnormal clone*	
PNH clone	Screen for glycosyl phosphatidyl inositol (GPI)-anchored proteins on red cells, granulocytes (and monocytes) using six-colour flow cytometry, including fluorescent aerolysin (FLAER). Size of PNH clone will help distinguish haemolytic PNH from AA/PNH and sub-clinical PNH. PNH clone detected in up to 40% of AA patients.
Cytogenetic clone using metaphase cytogenetics (MC) and cytoFISH or Single Nucleotide Polymoprhism-Array Karyotyping (SNP-A)	Detected in up to 12% of AA patients in the absence of dysplastic changes. Monosomy 7 is typical of MDS and AA which has evolved to MDS. MC may fail due to hypocellular BM and small number of metaphases. Other cytogenetic abnormalities (del13q, del20q, +8) can be seen in morphologically typical AA. SNP-A can detect copy neutral loss of hetrozygosity (CN-LOH), especially in 6p (HLA locus) and is also helpful in cases of failed MC
T-large granular lymphocyte (T-LGL) clone	If blood/BM T-lymphocytosis, perform *TCR* gene rearrangement to detect clonal T-LGL; in this situation, exclude *STAT3* mutation.
3. *Tests to exclude an inherited bone marrow failure syndrome*	
DEB test	PB chromosomal breakage (spontaneous and DEB/MMC-induced) increased in Fanconi anaemia. Screen patients up to 60 years old (FA has rarely been diagnosed in 5th decade) and all BMT candidates.
PB telomere length	Flow FISH or multiplex qPCR analysis. Very short telomeres (<1st percentile for age) are indicative of DC. Telomere gene mutation analysis then indicated (including *TERC*, *TERT*, *TIN2*, *DKC1*).
Other IBMFS	Specific gene mutation analysis when suspected, e.g. *SBDS* for Schwachmann–Diamond anaemia, *GATA2* for Emberger's syndrome. See point number 6 for emerging technologies to detect other IBMFS.
4. *Emerging diagnostic tests*	
Somatic gene mutation panel	Next-generation sequencing technology to detect somatic mutations which may help distinguish AA from hypocellular MDS/AML at diagnosis and during the disease course

(*Continued*)

Table 31.1 (*Continued*)

IBMFS custom array mutation analysis using exome/whole genome sequencing or targeted cytopenia panel (National Genomic Test Directory, R91 cytopenia panel)	To detect known (exome sequencing/targeted sequencing) or unknown (whole genome sequencing) IBMFS. See Chapter 8.
5. *How severe is the aplastic anaemia?*	
Severe AA (Camitta *et al*., 1976)	BM cellularity <25%, or 25–50% with <30% residual haemopoietic cells and 2 out of 3 of the following: *6. neutrophils* $<0.5\times10^9$*/L* *7. platelets* $<20\times10^9$*/L* *8. absolute reticulocyte count* $<60\times10^9$*/L*
Very severe AA (Bacigalupo *et al*. 1988)	As for severe AA but neutrophils $<0.2\times10^9$/L.
Non-severe (moderate) AA	Patients not fulfilling the criteria for severe or very severe aplastic anaemia.
6. *Is there an alternative diagnosis?*	
In the presence of pancytopenia and a hypocellular BM, exclude other possible causes:	
Hypocellular MDS/AML, and hypoALL in children	These features occur in hypoMDS/AML but not in AA: dysplastic neutrophils, dysgranulopoiesis and dysmegakayopoiesis, blasts in PB or BM, ring sideroblasts, increased BM reticulin and ALIP.
Hodgkin or Non-Hodgkin lymphoma	Examine trephine carefully for foci of lymphoma and perform immunophenotyping and gene rearrangement analyses.
Myelofibrosis	Presence of splenomegaly, tear-drop poikilocytes and leucoerythroblastic changes on blood film, and increased BM reticulin and/or collagen are all features of myelofibrosis.
Mycobacterial infection, especially atypical forms	Other BM features of mycobacterial infection are granulomas, necrosis and increased reticulin.
Anorexia nervosa, starvation	Often presents with neutropenia before pancytopenia. BM features include stromal oedema and serous degeneration.
Thrombocytopenic disorders, e.g. ITP, amegakaryocytic thrombocytopenia (AMT)	Sometimes AA can present with isolated thrombocytopenia. BM in ITP usually shows normal or increased megakaryocytes, but occasionally reduced megakaryocytes. AMT can later progress to AA or MDS. Congenital AMT is due to *c-MPL* mutation.

The immune-mediated nature of acquired AA

In acquired AA, it is proposed that an initiating event, such as a virus or drug, provokes an aberrant immune response, triggering oligoclonal expansion of cytotoxic T cells that destroy HSPCs (Figure 31.2). HSCT or IST leads to complete or partial response by eradicating or suppressing pathogenic T cell clones. Relapse occurs with recurrence of the immune response, and the immunologically stressed and depleted stem-cell compartment also allows selection of abnormal haemopoietic clones that manifest as MDS and occasionally AML.

Strong evidence that AA has an autoimmune nature is based on the following observations:

1 Haematological recovery after IST with antithymocyte globulin (ATG) and ciclosporin (CSA) occurs in the majority of patients.

2 There are activated autoreactive oligoclonal $CD8^+$ T cells present in blood and bone marrow that release IFN-γ, tumour necrosis factor (TNF)-α and other cytokines that inhibit haemopoiesis.

3 Intracellular IFN-γ levels in T cells correlate with response to IST.

4 Increased Fas expression on bone marrow $CD34^+$ cells indicates increased apoptosis.

5 T cell repertoire analysis shows oligoclonal expansion of $CD8^+$ T cells in AA, MDS and PNH.

6 Transcription factor T-bet is upregulated and binds to the IFN-γ promoter, resulting in increased expression of IFN-γ.

7 $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells are impaired and clonally restricted Th1 are expanded inducing an inflammatory microenvironment.

Gene expression profiling in AA $CD34^+$ cells has shown that more than half the upregulated genes are related to the immune response, including genes for cytokines and cytokine receptors and signal transduction genes, as well as other immune response genes. Many apoptosis and cell death genes are upregulated and some antiapoptotic genes downregulated.

It is unclear why T cells are activated in AA. HLA-DR2 and its split HLA-DR15 and DRB1*1501 and 1502 alleles are

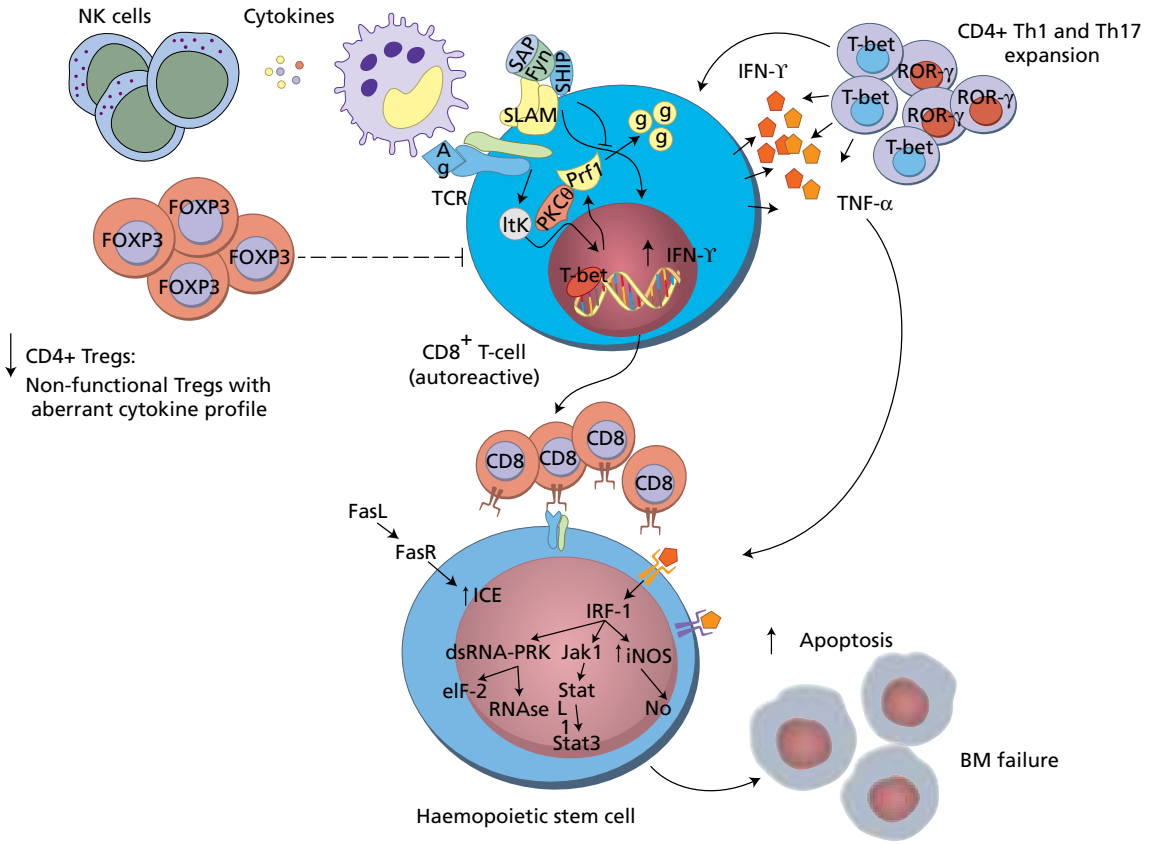


Figure 31.2 Immune-mediated aplastic anaemia. Following an insult, most likely viral, there is immune-mediated damage to stem cells, resulting in a pro-inflammatory response. The most important component comprises the $CD4^+$ T-cells, with expansion of Th1 (clonal) and Th17 cells, and reduced and dysfunctional regulatory T-cells (Tregs). This leads to oligoclonal expansion of cytotoxic $CD8^+$ T-cells, resulting in apoptotic death of stem cells (Source: Kordasti *et al.* 2012 [*Blood* **119**(9): 2033–43]. Reproduced with permission of American Society of Haematology.)

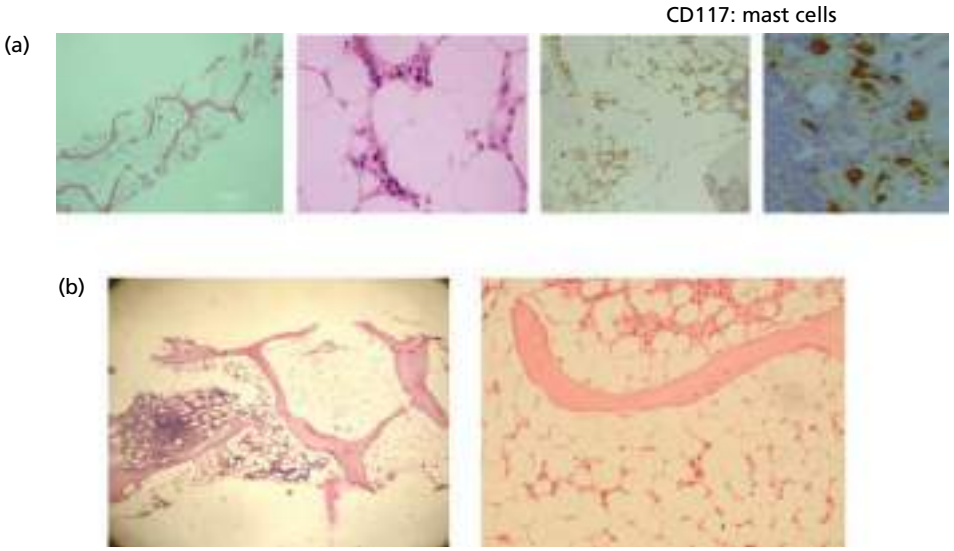


Figure 31.3 (a) BM trephine – severe AA; (b) Non-severe AA.

overrepresented in AA, as are class I HLA-B*4002 and HLA-A*0206, indicating a possible role for antigen recognition. There is correlation between ATG response and DRB1*1501, but most of these HLA data come from studies of Japanese patients. Loss of HLA expression, either by acquired copy neutral loss of heterozygosity (CN-LOH) on 6p or mutations on HLA loci, leads to escape of HSCs from activated cytotoxic T cells. Studies of cytokine gene polymorphisms that may reflect a heightened immune response in AA are limited; polymorphisms in the TNF-α promoter, IL-6 and IFN-γ genes have been reported, but a systematic assessment of all potentially relevant cytokine genes has not been reported.

The specific cytotoxic T-cell targets on HSPC in AA have not been identified. Potential candidates, identified by screening antibodies in patients' serum against a peptide library using leukaemia cell lines, include kinectin, DRS-1 (diazepam-binding inhibitor-related protein-1), PMS1 (post-meiotic segregation increased 1), moesin and hnRNPK (heterogeneous nuclear ribonucleoprotein K). However, the relevance of these findings is unclear, and they may represent epiphenomena rather than primary targets of cytotoxic T-cell attack.

Alternatively, a defect in the glycosylphosphatidylinositol (GPI) anchor may be the trigger for the immune response against normal progenitor cells through aberrant expression of intracellular GPI protein(s) while at the same time providing a protective mechanism for GPI-defective cells.

Detection of somatic mutations in AA

Using next-generation sequencing, two large studies have reported similar incidences (20–25%) of somatic (acquired) mutations in AA in genes associated with myeloid malignancies (e.g. *ASXL1, RUNX1* and *DNMT3A*). These mutations are predictive for later evolution to MDS/AML, especially in patients with long history of AA and also for worse survival following immunosuppressive therapy; in contrast, other mutations confer good prognosis (*BCOR, BCORL1* and *PIG-A*). Their further significance in AA is discussed later, but clonal haematopoiesis in AA seems ubiquitous and is present in a vast majority either as *PIG-A* mutations, cytogenetic abnormalities, loss of heterozygosisity of 6p locus or *HLA* mutations and somatic alterations of genes like *BCOR/BCORL1, DNMT3A* and *ASXL1* (see 'Suggested further reading').

Genetic predisposition to AA and short telomeres

Inherited forms of AA have long been regarded as very rare causes of acquired AA, including FA, DC and SDS (see Chapter 30 for detailed discussion). More recent awareness of these syndromes, with characterisation of the genetic mutations in these and other BMFS and the finding of very short telomeres in some patients with AA, indicate a more common occurrence than previously realised, and recognition that such cases may present later in adulthood.

The DC genes are involved in telomere maintenance, and all DC patients invariably have very short telomeres. About 10% of patients with apparent acquired AA have inherited mutations in *TERC*, *TERT*, with none or very mild mucocutaneous abnormalities. Families may have individuals with pulmonary fibrosis and hepatic cirrhosis, with or without cytopenia(s). SDS usually occurs in childhood with neutropenia but can present in adult life.

In addition to mutations in the DC genes, other factors such as 'stressed' haemopoiesis as a result of reduced stem cell numbers in AA, and environmental factors such as smoking, contribute to the shortened telomeres. Telomeres are assumed to be short in all organs, but they are only measured in blood leucocytes (the tissue for which there are sufficient age-matched controls). The consequences of telomere shortening include genomic instability, resulting in increased risk of malignant transformation (MDS, AML), bone marrow failure and defects in repair and regeneration (pulmonary fibrosis, cirrhosis). The clinical consequences of missing late-onset inherited forms of AA, as they often lack somatic anomalies, include likely mortality after allogeneic HSCT using standard conditioning regimens in FA due to the defect in DNA repair, and in DC due to the higher risk of liver and/or respiratory failure, and failure to screen potential family bone marrow donors for inherited bone marrow failure.

That not all patients with acquired AA respond to IST could be explained in pathogenetic terms by: (i) complete stem cell depletion due to a severe autoimmune attack, (ii) insufficient degree of IST and persistence of cytotoxic T cells or (iii) a non-immune basis for the bone marrow failure, of which one mechanism may be a genetic predisposition resulting in genomic instability. The presence of shortened telomeres in idiopathic AA does not preclude initial response to IST but predicts relapse after ATG and also late clonal disorders. There are insufficient data in patients with *TERC* or *TERT* mutations on response to IST (see also section 'Predictors of response to ATG').

Clinical features

Patients with AA most commonly present with symptoms of anaemia and skin or mucosal haemorrhage (ecchymoses or petechiae), including buccal haemorrhages, or visual disturbance due to retinal haemorrhage. Infection may be a presenting feature but is less common. There is no lymphadenopathy or hepatosplenomegaly in the absence of infection. An important careful history and clinical examination help to exclude an inherited form of AA, especially in children and young adults. This also applies to older patients as it has more recently been

realised that typical features of inherited BMFS may be absent and/or other features may be present in older patients. The findings of short stature, abnormal thumbs and forearms, café-au-lait spots and skeletal anomalies would raise the possibility of an inherited form of AA, specifically FA, although FA can present in adults without physical anomalies. Patients with FA most commonly present between the ages of 3 and 14 years, but can occasionally present later in their thirties or forties and very rarely early fifties. The findings of leucoplakia, nail dystrophy and pigmentation of the skin are characteristic of DC, with a median age at presentation of 7 years (range 6 months to 26 years). Some affected patients may have none of these clinical features and the diagnosis is made later after failure to respond to IST. For patients with AA and an inherited *TERC*/*TERT* mutation, the family history is very important as the pedigree may reveal pulmonary fibrosis, cirrhosis, osteoporosis, avascular necrosis of bone, low blood counts or cancer. A previous history of malabsorption or neutropenia may underlie a diagnosis of SDS in children or young adults, especially as the malabsorption often resolves in later life. A preceding history of jaundice, usually 2–3 months, strongly suggests sero-negative, posthepatitic AA.

Diagnostic investigations and differential diagnosis

To diagnose AA with certainty, it is important to consider other possible causes of pancytopenia with a hypocellular bone marrow and to exclude an inherited form of AA, as this will have important implications for treatment options, the choice of conditioning regimen and donor for HSCT, genetic screening and counselling of family members (Table 31.1). All patients should be screened by cytogenetics and for a PNH clone. See Table 31.1 and Figure 31.3 for summary of laboratory assessment of a newly presenting patient.

Management

General comments relating to the management of AA

Because AA is a rare disease, the haematologist responsible for the patient should contact a centre/specialist with expertise in AA soon after presentation to discuss a management plan for the patient. Patients should be offered the opportunity to be reviewed in a specialist centre. Whenever possible, patients should be enrolled into prospective national or international trials.

Prior to administration of specific treatment for the disease, the patient should be stabilised clinically in terms of controlling bleeding and treating infection. The presence of infection is an adverse factor for outcome after HSCT, although it may sometimes be necessary to proceed with HSCT in the presence of active infection, particularly fungal infection. In this setting, transplantation may offer the best chance of early neutrophil recovery, and delaying the transplant may risk progression of the fungal infection.

Supportive care

Transfusions

Support with red cell and platelet transfusions is essential for patients with AA to maintain a safe blood count. Current practice is to give prophylactic platelets when the platelet count is below 10×10^9/L (or higher in the presence of fever, sepsis or bleeding). Fatal haemorrhage, usually cerebral, is more common in patients who have less than 10×10^9/L platelets, extensive retinal haemorrhages, buccal haemorrhages or rapidly spreading purpura. Other important practical measures to help prevent bleeding include good dental hygiene, use of antifibrinolytics (e.g. tranexamic acid) and control of menorrhagia with appropriate hormone therapy.

A common problem in multitransfused AA patients is alloimmunisation to leucocytes present in red cell and platelet transfusions by generating HLA or non-HLA (minor histocompatibility) antibodies. Universal leucodepletion has probably reduced, but not eliminated the incidence of alloimmunisation; the incidence in AA was around 30–50% in the preleucodepletion era. Alloimmunisation can result in platelet refractoriness, as well as an increased risk of graft rejection after allogeneic BMT. Patients who become refractory to platelet transfusions should be screened for HLA antibodies, once other non-immune causes have been excluded, and if present HLA-matched platelets should be used. Novel approaches, including the use of HLA epitopematched platelets, are currently being evaluated in clinical trials in view of the shrinking HLA-typed donor pool.

Platelet transfusions should be given to maintain a safe platelet count and blood transfusions to allow normal daily activities. Transfusions should not be withheld for fear of sensitising the patient. Directed blood and platelet donations from family members should be avoided, as the recipient may become sensitised to minor histocompatibility antigens from the potential bone marrow donor, increasing the risk of graft rejection.

Even though there is no evidence base, the British Committee for Standards in Haematology now recommends empirically the use of irradiated blood components for all AA patients receiving ATG. The rationale for this is twofold. Firstly, animal data demonstrate that irradiation of all red cell and platelet transfusions before HSCT in addition to leucodepletion, further reduces the risk of sensitisation to minor histocompatibility antigens (and hence reduced risk of graft rejection after allogeneic HSCT). Secondly, irradiated blood products would abolish the potential risk of transfusion-associated GVHD associated with ATG therapy. Transfusion-associated GVHD has very rarely been reported after ATG.

Irradiated granulocyte transfusions are used to support patients with life-threatening neutropenia, especially with active fungal/mould disease or bacterial sepsis and not adequately responding to maximal antimicrobials. Adverse events, such as febrile reactions, HLA alloimmunisation and transfusion-related acute lung injury, are well-recognised complications following granulocyte transfusions.

Iron chelation therapy

Many AA patients require multiple red cell transfusions leading to transfusion haemosiderosis. Iron overload impacts adversely on outcome after allogeneic BMT. Iron chelation therapy should commence when the serum ferritin is above 1000 µg/L, although the evidence base for this is lacking. It may be difficult to deliver subcutaneous desferrioxamine to AA patients on account of local haemorrhage and infection from subcutaneous injections. Alternative oral agents include deferiprone and deferasirox (Exjade). Because of a high incidence of agranulocytosis with deferiprone, it is not used routinely in patients with AA. Results of a recent prospective study of deferasirox in 166 iron-overloaded AA patients showed the safety and also the ability to reduce ferritin (median fall in serum ferritin of 946 µg/L), although in view of renal toxicity of the drug, extra vigilance should be used in those on CSA.

Infections

Patients with AA are at risk of bacterial and fungal infections, depending in part on the degree of neutropenia. Very severe neutropenia (neutrophils $<0.2 \times 10^9$/L) carries a high risk. If there is severe neutropenia, either prophylactic non-absorbable antibiotics or oral quinolone are administered to reduce the potential pathogenic load from the gastrointestinal tract, although neither is standard practice in the USA. Oral hygiene is important. Entry sites for venous access are potential sources of systemic infection. Patients with AA are at high risk of fungal infection, including *Aspergillus*. Fluconazole provides no protection against *Aspergillus* species, for which the drugs of choice are itraconazole, which has clinically significant, but manageable or avoidable interactions with other drugs, and posaconazole, which has not yet been shown to be superior in efficacy to itraconazole in this setting.

Fever should be treated with broad-spectrum antibiotics without waiting for laboratory isolation of organisms, and with early introduction of systemic antifungal therapy if fever fails to respond to antibiotics. Chest radiography and CT scan of chest and sinuses should be part of the investigation of new or persistent fever. G-CSF is usually ineffective in severe AA due to a marked reduction or absence of myeloid progenitor cells, although it is reasonable to consider giving a short course in life-threatening infections.

Indications for treatment of AA

The two main treatment options for AA are allogeneic HSCT or IST with ATG and CSA, based on disease severity, age of the patient, availability of a matched sibling donor, patient comorbidities and patient choice (Figure 31.4). For patients with severe AA who are under 50 years of age, the first-line treatment is matched sibling donor HSCT. EBMT data show similar outcomes for patients aged 40–50 to those aged 30–40 years. However, comorbidities should be carefully assessed to determine fitness for up-front transplant instead of immunosuppressive therapy for patients, especially those aged 40–50 years. For all other patients who require treatment, namely those lacking HLA compatible sibling donor, non-severe AA or those older than >50 years, IST is the first option. Unrelated donor HSCT is indicated for adults after failure to respond to one course of IST, for those patients who lack a matched sibling donor. Upfront MUD HSCT may be considered in children who lack a MSD if a suitable donor is readily available.

The standard regimen for IST comprises the combination of horse ATG and CSA, although addition of Eltrombopag to IST for first-line treatment of SAA is becoming standard practice. This was initially based on single-arm study demonstrating better outcome and with more robust and early haematological response, included high complete response. This data was confirmed in a large prospective randomised controlled study comparing standard IST (horse ATG and CSA) with eltrombopag added to standard IST. The triple drug combination improved the rate, rapidity and strength of haematological response, without additional toxicities.

Corticosteroids should not be given to treat AA, except to prevent and treat serum sickness, as they are ineffective and encourage fungal and bacterial infections.

Immunosuppressive therapy

Antithymocyte globulin: properties, mechanism of action and administration

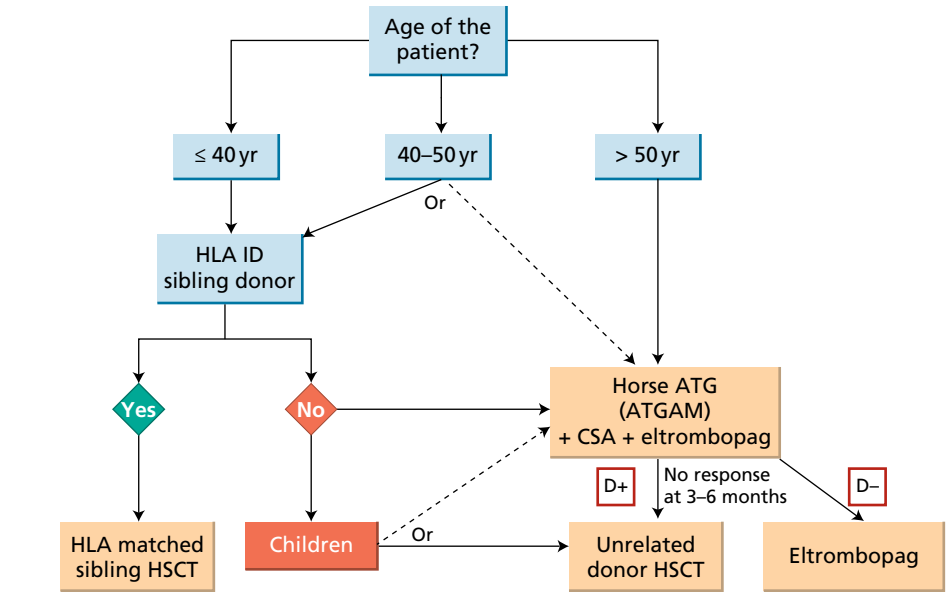
ATG is a polyclonal IgG antibody preparation produced by immunising horses or rabbits with human thymocytes. Its mechanism of action in AA is not entirely clear. Possible mechanisms include the following:

1 T cell depletion by complement-mediated lysis;

2 destruction of activated cytotoxic T lymphocytes by Fas-mediated apoptosis and antibody-dependent cellular cytotoxicity;

3 reduced apoptosis and Fas expression on AA $CD34^+$ bone marrow cells;

4 direct stimulation of normal and AA $CD34^+$ bone marrow cells and a mitogenic effect in the absence of complement resulting in release of HGFs;



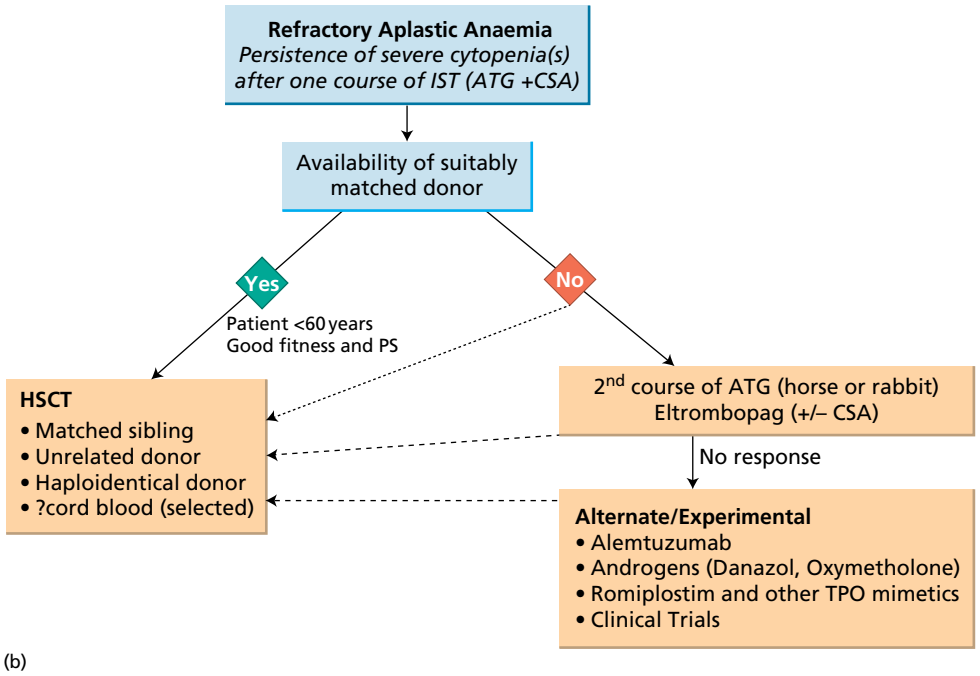


Figure 31.4 (a) Treatment of severe aplastic anaemia. (b) treatment of refractory severe aplastic anaemia. For patients age >50 years, who fail to respond to first-line IST, HSCT may be considered, using a matched sibling donor, or a suitably matched unrelated donor if no matched sibling is available.

5 direct stimulation of T regulatory cells has been demonstrated when normal bone marrow mononuclear cells are incubated with rabbit ATG.

In the UK, most of Europe and many other countries, the standard preparation of ATG has until recently been horse ATG (Lymphoglobuline, Genzyme). The rabbit preparation (Thymoglobuline, Genzyme) was usually reserved for second or subsequent courses. From June 2007, horse ATG (Lymphoglobuline) was withdrawn due to manufacturing difficulties in maintaining quality control, but another preparation of horse ATG (ATGAM, Pfizer) is now commonly used. Horse ATG is preferred to rabbit ATG as it results in a higher response rate (68% at 6 months compared to 37% for rabbit ATG, Table 31.2) and better survival, despite rabbit ATG being more immunosuppressive than horse ATG in terms of the degree and duration of lymphodepletion, indicating that different mechanisms are important in the mode of action of the two agents.

Table 31.2 Direct comparisons of horse and rabbit ATG with CSA as first-line treatment for aplastic anaemia in the last 10 years.

		N, preparation		Response at 6 months		Overall survival		
	Study design	**Horse ATG**	**Rabbit ATG**	**Horse ATG**	**Rabbit ATG**	**Horse ATG**	**Rabbit ATG**	**Comments**
Scheinberg, 2011	Prospective, randomised	60, ATGAM	60, Thymoglobulin	68%	37%	96%	76% at 3yr	Significant difference between ATG preparations
Marsh, 2012	Prospective	105, Lymphoglobulin	35, Thymoglobulin	67%	40%	86%	68% at 2yr	Matched pair analysis between two preparations; Significant differences between ATGs. More infective deaths with rabbit ATG.
Jeong, 2014	Retrospective	297, Lymphoglobulin	158, Thymoglobulin	60%	55%	96%	87%	Study in children. Significant difference in survival but not response. More infections with rabbit ATG.
Afable, 2011	Retrospective	67, ATGAM	20, Thymoglobulin	58%	45%	76%	64% at 2yr	No significant difference between ATGs but study likely under-powered.
Atta, 2010	Retrospective	42, Lymphoglobulin	29, Thymoglobulin	60%	35%	78%	55% at 2yr	Significant difference between ATG preparations.
Chen, 2013	Retrospective	42, Lymphoglobulin	53, Thymoglobulin	39%	45%	84%	83%	No significant difference between ATGs

ATG is a powerful immunosuppressive drug, given as an inpatient treatment and requires very careful monitoring, the prophylaxis and treatment of fevers and infections, as well as adequate (and sometimes intensive) platelet transfusion support. ATG is given as a daily intravenous infusion usually over 12–18 hours through a central venous catheter. Instead of giving a separate test dose, most centres give the first 100 mL of the first infusion very slowly over 1 hour to assess for possible anaphylaxis. Allergists maintain that this is inadequate to prevent anaphylaxis as the amounts delivered are relatively large. The dosage of horse ATG (ATGAM) is 40 mg/kg daily for 4 days and rabbit ATG is 3.75 mg/kg for 5 days.

Immediate side-effects are allergic and occur commonly, including fever, rigours, rash, hypertension or hypotension and fluid retention. Each daily dose should be preceded by intravenous methylprednisolone and chlorpheniramine. In the USA, patients are pretreated with diphenhydramine and pethidine if fever and chills have occurred. Platelet transfusions should be given to maintain a safe platelet count (ideally $>30\times10^9$/L; in the USA, a threshold of $>20\times10^9$/L is used). Prior to starting ATG, patients should be assessed to ensure adequate platelet increments with random donor platelets. Poor platelet increments should be investigated beforehand. Patients are often in isolation with reverse barrier nursing, but this is not standard practice in the USA. Fevers are treated with broad-spectrum antibiotics. Intravenous methylprednisolone (1–2 mg/kg) (or oral prednisolone) and paracetamol are given at least 30 minutes before each daily dose of ATG with reduction of the prednisolone dose by half every 5 days. Prednisolone is given to help prevent serum sickness. Serum sickness typically occurs between 7 and 14 days from the start of ATG treatment. The common symptoms of serum sickness include arthralgia, myalgia, rash, fever, mild proteinuria and platelet consumption, often necessitating increased platelet transfusion support.

There are no indications for routine use of daily G-CSF (administered for 3 months) with ATG and CSA as three prospective randomised studies comparing ATG, CSA and G-CSF with ATG and CSA alone demonstrated no difference in response and survival between the two groups, and there is concern about G-CSF stimulating low-level monosomy 7 clones. Attempts to augment responses and reduce relapse by incorporation of high-dose corticosteroids, mycophenolate mofetil (MMF), sirolimus or androgens to standard IST (ATG and CSA) have not met with any success.

Survival after ATG

Data from EBMT and large single centres confirm that over the last three decades overall survival after ATG therapy has improved steadily, with 5-year survival of around 80% reported (Table 31.2). The reasons for improved survival have been difficult to assess formally, but likely factors include: (i) improved supportive care for prevention and treatment of infections, especially superior antifungals and better availability and quality of blood products, (ii) the use of CSA with ATG, (iii) addition of eltrombopag in the frontline setting to ATG and CSA and (iv) successful second-line and rescue therapies, including another round of ATG, eltrombopag, unrelated donor (UD) HSCT in younger patients and allogeneic HSCT in older adults (see also Chapter 37).

Predictors of response to ATG

Patients with non-severe AA are more likely to respond than are patients with severe or very severe AA. Other predictors are younger age, absolute reticulocyte count (ARC) $>25\times10^9$/L and absolute lymphocyte count (ALC) $>1\times10^9$/L, which correlate with response to ATG. Same factors (age, disease severity and ARC) remain predictors of response for IST combined with Eltrombopag.

Several studies have examined whether the presence of a PNH clone is associated with response to ATG, with conflicting results. This may reflect differences in sensitivity of the test used to detect a PNH clone. Those using very sensitive tests to detect GPI-deficient clones of less than 0.003 cells show a strong correlation with response.

HLA type may also correlate with response to immunosuppressive therapy. A Japanese study showed that HLA-DRB1*1501 associated with response to CSA. Although HLA-DR15 or DRB1*1501 predicts response to ATG in white patients with MDS, no association was seen in AA patients. More recently, the same Japanese group demonstrated that of the 30 different DRB1 alleles, only DRB1*1501 and DRB1*1502 occurred more frequently in AA than in controls, and that response to ATG and CSA was only associated with DRB1*1501 in the presence of a PNH clone. Thus specific HLA haplotypes correlate with response to immunosuppressive therapy in Japanese patients, but similar studies in white Europeans are lacking.

Recent studies from NIH indicate that shortened telomere length does not preclude initial response to immunosuppressive therapy, but predicts relapse after ATG and is a risk factor for later cytogenetic abnormalities and evolution to MDS and AML. Thus telomere length at diagnosis may reflect depleted stem cell reserve so that prolonged stem cell division is not possible, resulting in later relapse; later clonal evolution reflects genomic instability of the critically shortened telomeres.

Repeat courses of ATG

A second course of immunosuppression may be administered (horse ATG, rabbit ATG or alemtuzumab). The response rate following a second course of ATG (horse or rabbit) for relapse is around 60%, but only about 35% for patients who did not respond to the first course. A study from Japan examined prospectively the outcome of 52 children who failed one course of horse ATG, and who went on to receive either a second course of horse ATG or an unrelated donor HSCT. The response to a

second course of ATG was only 11%, with a 5-year failure-free survival of only 9.5%. Another study has assessed the value of giving three courses of ATG. Amongst those patients who showed no response to the first or second courses, there were no sustained responses, but for those who had relapsed after two previous courses, all responded to a third. For patients receiving more than one course of ATG, the risk of anaphylaxis may be higher. Serum sickness is still unpredictable, though it may occur earlier following a second course of the same animal preparation of ATG.

Late complications after ATG

Relapse occurs in up to a third of patients when CSA is withdrawn at 6 months. A more prolonged course of CSA with a later slow tapering of the drug reduces the relapse risk to around 13–16%, although data from NIH show that a long course of CSA only delays but does not prevent relapse. About one-third of patients are CSA dependent and require a small dose long term. CSA can be continued for at least 12 months after a maximal response before starting to taper the drug, followed by a very slow taper, for example by 25 mg every 3 months.

Patients treated with ATG are also at risk of developing clonal disorders, such as PNH, MDS and AML. Long-term follow-up from a large NIH cohort showed 35% relapse at 5 years, and 10% risk of PNH and 15% risk of clonal evolution, excluding PNH, at 10 years. A previous study from NIH showed 20% risk at 10 years of clonal cytogenetic abnormalities after ATG. Reported risk factors for developing MDS/AML include repeated courses of ATG, older age, and high doses, prolonged duration of G-CSF with ATG and CSA, shortened telomeres and presence of acquired somatic mutations in genes known to be mutated in MDS/AML.

Although data is still not mature for long-term complications of eltrombopag used in combination with ATG, relapse and clonal evolution to MDS/AML were similar to IST alone but occurred earlier in the disease course

ATG treatment in older patients and children

For patients >60 years old, the response rate and survival rate are lower compared with younger patients, with a higher risk of bacterial and fungal infections, bleeding and significant cardiac events after ATG. Although there is no upper age limit for ATG treatment, consideration for treatment should be preceded by medical assessment to exclude significant comorbidities and hypocellular MDS. For older patients who are not candidates for ATG, CSA may be considered, but there is increased risk of renal toxicity and hypertension.

Excellent response to ATG is seen in children. Current response rates are around 75%, with 90% long-term survival, but issues of relapse and clonal evolution persist. In view of excellent outcomes after MUD HSCT in children using alemtuzumab-based regimens, upfront MUD HSCT may be considered instead of ATG when an MSD is not available. Eltrombopag with ATG did not seem to improve the response rate in children and hence additional rationale for upfront HSCT.

Other drugs used in AA Cyclophosphamide

High-dose (200 mg/kg) and recently 'moderate-dose' (120 mg/kg) cyclophosphamide (Cy) has been used to treat AA in the absence of stem cell support, following the observation that complete autologous haematological recovery occurs in a small number of patients undergoing allogeneic sibling BMT using Cy. Although durable responses were seen, the predictable and markedly prolonged cytopenias exposed patients to a high risk of fatal fungal infections and a significant increase in use of blood and platelet transfusions, days of intravenous antibiotics and amphotericin, and inpatient days in hospital. In addition, HDC treatment does not eliminate the risk of clonal events or relapse. Consequently, the use of Cy in the treatment of AA is not recommended.

Alemtuzumab

The anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) is effective in other autoimmune disorders such as multiple sclerosis, autoimmune cytopenias and the vasculitides. NIH investigated the use of alemtuzumab monotherapy (10 mg intravenous for 10 days) without CSA in AA and induced heterogeneous responses; 56% in relapsed setting, 37% in refractory disease and 19% in treatment-naïve AA. Alemtuzumab can alternatively be given subcutaneously. The ease of administration, its efficacy even without concurrent use of CSA and the relatively good safety profile, makes alemtuzumab an alternative choice of IST in relapsed/refractory AA for salvaging transplant-ineligible patients.

Eltrombopag

Eltrombopag, an oral thrombopoietin mimetic, licensed in chronic immune thrombocytopenic purpura, induces platelet maturation and release by binding to c-MPL receptors on megakaryocytes (Chapters 40 and 45). In refractory severe AA, eltrombopag induced responses in 40% patients; responses included tri/bilineage, were robust and led to normalisation of cellularity. Trilineage responses, although surprising, might indicate stimulation of c-MPL receptors on remaining stem cells. The drug was well tolerated and there were no reports of increased reticulin or collagen fibrosis in the bone marrow, although new cytogenetic abnormalities, especially monosomy 7, were detected. The safety of eltrombopag needs to be evaluated further in prospective clinical trials, especially in view of the possible link to clonal evolution in refractory AA. The response seen with eltrombopag in refractory disease has led to design of prospective trials with incorporation of this agent along with ATG+CSA for first-line treatment of AA with remarkable improvement in outcome. Eltrombopag has now had FDA

approval for refractory SAA and also in combination with ATG and CSA in treatment naïve SAA.

Androgens

Androgens, such as oxymetholone, have been used historically for the treatment of AA prior to availability of ATG/CSA, subsequently as an adjunct to ATG and are still used in certain developing countries for first-line treatment of AA. Androgens lead to increased telomerase activity via aromatisation of oestradiol to steroids and hence induce responses in patients with telomeropathies who manifest as apparent acquired AA.

Haemopoietic stem cell transplantation

Outcomes following allogeneic HSCT for acquired AA, whether from a matched sibling donor (MSD) or a matched unrelated donor (MUD), have steadily improved over time. More recently, significant improvements have been observed for matched unrelated donor HSCT for AA patients failing immunosuppressive therapy, with 80–90% survival and the best results seen in younger patients (see also Chapter 37).

Allogeneic HSCT

Conditioning regimens

For MSD HSCT, the standard immunosuppressive, nonmyeloablative conditioning regimen for patients <30 years old is high-dose cyclophosphamide 200 mg/kg along with ATG, which results in survival of at least 80% in younger patients. For patients >30 years old, a fludarabine-based regimen is used, with a lower dose of cyclophosphamide, with either ATG ('FCATG') or alemtuzumab ('FCC'), to reduce rejection and GVHD, and improve outcomes. Alemtuzumab is associated with a lower incidence of GVHD, especially chronic GVHD compared to ATG. Standard GVHD prophylaxis is with CSA and methotrexate, although CSA alone is sufficient in the setting of alemtuzumab. Because AA patients are at risk of late graft failure, CSA is usually continued for 12 months after HSCT.

For MUD HSCT, the conditioning regimen is identical to that used for older patients undergoing MSD HSCT using either FCATG, with addition of low dose (2Gy) TBI, or FCC without TBI. Recent studies using alemtuzumab-based conditioning show 90% OS in children and around 80% in adults; and 70% in patients >50 years old. High-resolution HLA tissue typing is now routine for selection of MUDs.

Dose and source of haemopoietic stem cells

It is important to give at least 3×10^8 nucleated marrow cells/kg and $>2 \times 10^6$ $CD34^+$ cells/kg, because lower doses delay neutrophil engraftment and increase the risk of graft rejection. The strongly preferred stem cell source is BM for ATG-containing regimens, due to higher incidence of chronic GVHD and worse survival with PBSC, but either BM or PBSC can be used for alemtuzumab-based conditioning.

Chimerism

Using polymerase chain reaction for short tandem repeats on unfractionated bone marrow or peripheral blood mononuclear cells, the Dublin group, in collaboration with EBMT, reported mixed chimerism in 25% of HSCTs for AA. Patients with mixed chimerism may have either stable or progressive mixed chimerism. All the cases of graft failure occurred in the progressive mixed chimerism group, of whom 50% rejected their grafts. More often now, chimerism is examined in T-cells (CD3) and myeloid cells (CD15) and most patients have stable mixed T-cell chimerism with full donor myeloid chimerism when using alemtuzumab-based conditioning, with excellent survival and very low incidence of chronic GVHD, suggesting a state of tolerance may be achieved.

Graft-versus-host disease

GVHD (especially chronic) is one of the most serious complications, as it adversely impacts performance status, quality of life and survival after marrow transplantation, especially in a disease like AA where there is no advantage in having a graft versus-disease effect (Table 31.3), but the risk is reduced when using alemtuzumab-based conditioning.

The risk factors for chronic GVHD include: (i) history of prior acute GVHD, (ii) infusion of non-irradiated donor buffy coat cells (this manoeuvre was used previously to overcome the problem of graft rejection, but was abandoned because of the high incidence of chronic GVHD), (iii) older patient age, (iv) the use of peripheral blood stem cells and (v) the use of ATG compared with alemtuzumab-based conditioning regimens.

Long-term complications of HSCT

Fertility is usually well preserved when irradiation is not used in the conditioning regimen, and the chances of pregnancy or fathering a child are much higher in patients who receive a marrow transplant for AA than for haematological malignancies. Pregnancies in patients transplanted for AA usually have a successful outcome. Less long-term data are available using fludarabine with lower-dose Cy regimens, although cases of successful pregnancy have been reported. For patients of child-bearing age, referral to an assisted conception unit for discussions on fertility should be offered. Men should be offered sperm storage.

There is an increased frequency of solid tumours after HSCT for AA, but the risk is lower than in patients transplanted for haematological malignancies when irradiation is avoided. The

Table 31.3 Outcomes of HLA-matched sibling donor HSCT from recent studies using high dose cyclophosphamide (Cy) ± antithymocyte globulin (ATG) conditioning.

Study	Number of patients	Conditioning	Follow-up (years)	Overall survival	Acute GVHD	Chronic GVHD	Graft failure
Bacigalupo, 2012 EBMT study	1886	Cy±ATG predominantly		BM: 92% PB: 80%	BM: 11% PB: 17% (Gd II-IV)	BM: 11% PB: 22%	BM: 5.5% PB: 7.2%
Konopacki, 2012 Paris study	61	Cy+ATG	6 (0.6–19)	85% at 6 yr	23% Gd II-IV	32% CI	3.2%
Sanders, 2011 Seattle study, children	118	Cy±ATG	22 (1–38)	82% at 30 yr	30% Gd II-III	30%	N/A; autologous recovery in 2
Gupta, 2010, CIBMTR study	1307	Cy±ATG predominantly		Age <20: 82% 20-40: 72% >40: 53% at 5 yr	11% 17% 27%	11% 25% 27%	17% (lack of 13% neutr. 12% recovery)

ATG, antithymosyte globulin; BM, bone marrow; CIBMTR, Centre for International Blood and Marrow Transplant Research; Cy, ciclosporin; EBMT, European Group for Blood and Marrow Transplantation; gd, grade; N/A, not available; PB, peripheral blood.

risk is also lower in the absence of chronic GVHD. Monitoring of late effects should follow international guidelines, including routine surveillance for secondary malignancy, endocrine, metabolic, bone (including avascular necrosis) and cardiovascular risks.

Alternative donor HSCT

Umbilical cord blood transplantation

Experience in acquired AA is still limited. The largest study of unrelated cord blood transplantation (CBT) in acquired AA is reported by the combined EUROCORD/EBMT SAAWP. The main problem was engraftment failure in around 50% patients. A reduced intensity conditioning regimen is used. Because of the high risk of non-engraftment, a higher cell dose (EBMT recommendation is to use $>4 \times 10^7$ TNC/kg) is required compared to doses used in CBT for leukaemia and with no more than 2/6 HLA mismatches in the cord unit(s). As for haploidentical HSCT (see below), all patients must be screened for HLA antibodies that may be directed against HLA antigens present on the cord units and thereby increase the risk of rejection, so that only cord units lacking that antigen(s) are used.

Haploidentical HSCT

The key advantages of haploidentical HSCT are that a graft is available for most patients, the time to procure the graft is short and the cost is low. A novel approach is reduced intensity conditioning with high dose Cy given on day + 3 and + 4 posttransplant to prevent GVHD by depleting alloreactive donor T cells, but sparing quiescent, non-alloreactive T cells. This promising approach is undergoing further evaluation in AA and early data from prospective studies are encouraging.

Paroxysmal nocturnal haemoglobinuria

Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) has fascinated haematologists since its first definitive description in 1882 by Paul Strübbing. PNH is unique as an acquired haemolytic disorder in which the defect is intrinsic to the red cell. Patients have a propensity to develop thromboses that are frequently life-threatening. PNH is also associated with bone marrow failure and indeed may provide a unique insight into the pathophysiology of a variety of BMFS. Development of the terminal complement inhibitor eculizumab has revolutionised the treatment of PNH, but in turn, has revealed further insights into the pathophysiology of the disease and into normal physiology. The treatment of PNH with eculizumab is not without problems in that a minority of patients have suboptimal responses and require modifications to maximise the benefits of therapy. Novel complement blockade with different targets in the complement pathway and easier modes of administration is revolutionising the field of complement therapeutics, with proof of concept studies done in PNH and two agents (ravulizumab and pegcetacoplan) recently approved for use in PNH

Pathophysiology

Glycosylphosphatidylinositol defect

PNH results from the expansion of an abnormal haemopoietic clone that arises following an acquired mutation in a gene critical for the biosynthesis of GPI structures. These highly conserved GPI anchors are glycolipid structures through which a large number of cell-surface antigens are attached to the cell membrane. Almost all patients with PNH have the same biosynthetic defect in one of the early steps of the pathway, namely the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to phosphatidylinositol. In 1993, Miyata and colleagues reported the cloning of the phosphatidylinositol glycan complementation class A (*PIGA*) gene, which is part of the enzyme complex involved in this step of the pathway and which has subsequently been shown to be mutated in almost all cases of acquired PNH reported to date.

Mutations of the *PIGA* gene are different between patients as they are acquired not inherited. It appears that the mutation rate of *PIGA* is similar to that of other genes and in fact, populations of PNH-like GPI-deficient cells can be observed at extremely low levels (1–10 per million cells) in most normal individuals, indicating that occasional haemopoietic stem cells by chance acquire *PIGA* mutations. Since the *PIGA* gene is located on the X chromosome (Xp22.1), each somatic cell, male or female, only has a single active copy and therefore a somatic mutation of this active gene leads to deficiency of GPI biosynthesis. GPI-deficient clones only expand if there is an additional factor that encourages their selection. The mechanism of this selective growth advantage of PNH clones, which could be a second genetic event, is of key importance in understanding the pathophysiology of PNH. A much more likely explanation is indicated by the association between PNH and other BMFS.

Association with bone marrow failure

PNH clones are detectable in up to 50% of patients withAA and in a smaller proportion of patients with myelodysplastic syndrome. This suggests that such BMFS are permissive for the expansion of PNH clones. There is overwhelming evidence that AA is an autoimmune disorder in which there is an aberrant immune attack, probably by CD8 cytotoxic T-cells directed against the haemopoietic stem cell. It would therefore appear likely that PNH stem cells, presumably due to GPI deficiency, evade this immune attack. This implies that either the immune attack in BMFS is, at least in part, directed through a GPI-linked structure or that the biology of the PNH stem cell is abnormal, protecting it from immune-mediated attack. Understanding PNH may well provide an insight into the mechanism of a variety of BMFS and provide a unique therapeutic target for these conditions.

Epidemiology

If PNH is defined as the presence of a PNH clone identified during the investigation of bone marrow failure, haemolysis or thrombosis, then the incidence of PNH in a series from the UK was found to be 1.3 newly diagnosed patients per million per year, and the prevalence 15.9 patients per million population. However, of these patients, 57% have less than 10% PNH neutrophils, indicating that they are unlikely to have clinically significant haemolysis and only one-third reported macroscopic haemoglobinuria. However, the identification of the PNH clone is important, even when it is small, as it suggests that the bone marrow failure is likely to be immune-mediated and because the PNH clone can evolve over time so that patients may be at risk of haemolysis and/or thrombosis.

Clinical features

Clinical triad

The clinical picture in PNH depends on the balance between three components: intravascular haemolysis (IVH), bone marrow failure and thrombotic complications. The chronicity of the disease, with a median survival of 10–20 years even prior to targeted therapy, causes continuous, high-level IVH leading to other complications, including renal disease and cholelithiasis.

Haemolysis

PNH, the disorder with perhaps the most intense chronic IVH, is characterised by episodes of haemoglobinuria, during which the patient's urine is often black and which, for unknown reasons, is often worse in the morning. This episodic haemoglobinuria is frequently associated with disabling symptoms including profound lethargy out of keeping with the patient's anaemia, abdominal pain sometimes requiring opiates, dysphagia that can be temporarily absolute and erectile dysfunction. The degree of haemolysis is associated with the size of the PNH clone and particularly the proportion of PNH type III (completely deficient) red cells. Macroscopic haemoglobinuria is rarely seen unless there are at least 10% type III red cells. However, patients with apparently identical PNH clones can have a wide range of disease severity, from very occasional attacks of haemoglobinuria without anaemia to severe recurrent or even continuous haemoglobinuria and transfusion dependence, suggesting that other important factors influence the severity of the symptoms. It is possible that this variation is due to the severity of underlying bone marrow failure, to an inherent variation in complement activity or to some other unidentified factor.

Many of the symptoms and complications of PNH result directly from IVH. Free plasma haemoglobin is immediately bound to haptoglobin and removed, explaining why haptoglobin is depleted in every patient. Free haemoglobin binds to and removes other gases such as nitric oxide (NO). This depletion of NO in PNH results in smooth muscle dysfunction, vasoconstriction, and pulmonary and systemic hypertension. This leads to dysphagia, abdominal discomfort, possibly due to intestinal spasm, erectile dysfunction, severe lethargy, pulmonary hypertension and possibly thrombosis, all classic

symptoms of PNH. This syndrome of NO consumption has only become clear since the development of eculizumab (see below), which stops intravascular haemolysis in almost all patients and abrogates the clinical features of PNH.

Bone marrow failure

The degree of anaemia and other cytopenias is a composite of the activity of IVH and the degree of underlying bone marrow failure. The platelet count in many patients is a suitable surrogate for marrow function, but in some patients, for example those with previous intra-abdominal thrombosis and subsequent hypersplenism, there may be other causes for a low platelet count. The presence of a PNH clone in patients with bone marrow failure probably indicates that there is a significant immune component to the marrow failure and may suggest that immune suppression is a reasonable therapeutic option. The degree of bone marrow failure will also impact on the efficacy of eculizumab, as this will only treat the component due to complement activity, such as IVH and thrombosis.

Thrombosis

Thrombosis occurs in 40–50% of patients with haemolytic PNH, with a predilection for certain veins, such as the hepatic veins (Budd–Chiari syndrome), other intra-abdominal veins or the cerebral veins (Table 31.4). Approximately 10% of patients present with a thrombosis as their initial clinical manifestation of PNH. Recent data indicate that the thrombotic tendency in PNH is in arterial as well as venous sites, including cerebrovascular events and myocardial infarctions at an early age. After the first thrombosis, patients have a 7.8-fold increased risk of dying compared with those patients who have not had a thrombosis. A classical clinical scenario is that of downward spiralling thrombotic events: after a first thrombosis, patients continue to experience further apparently discrete thromboses despite what would be considered adequate anticoagulation with warfarin and/or heparin until they eventually succumb. The cause of thrombosis in PNH is multifactorial including activation of PNH platelets by complement as well as IVH and NO consumption, which may lead to endothelial damage and thrombosis. It is now clear that there is a close relationship between the activation of both coagulation and complement with thrombin directly activating complement through the alternative pathway. This explains why frequently patients enter a spiral of multiple and increasingly severe thrombotic complications as the first thrombosis leads to complement activation leading to further thrombosis and more thrombin generation. Anticoagulants are relatively ineffective at preventing this circle of catastrophic thrombosis as they do not stop the activation of complement, whereas eculizumab stops the further activation of complement and usually interrupts this vicious circle of thrombosis.

Table 31.4 Sites of thrombotic events in haemolytic PNH.

Venous thrombosis	
Deep vein thrombosis	41 (33.1%)
Lower extremity	23 (18.5%)
Other	18 (14.5%)
Mesenteric/splenic vein thrombosis	23 (18.5%)
Hepatic/portal vein thrombosis	21 (16.9%)
Pulmonary embolus	8 (6.5%)
Cerebral/internal jugular thrombosis	7 (5.6%)
Superficial vein thrombosis	5 (4.0%)
Arterial thrombosis	
Cerebrovascular accident/TIA	17 (13.7%)
Myocardial infarction/unstable angina	2 (1.6%)
Total	124 (100%)

(*Source:* Hillmen *et al.* 2007 [*Blood* 2007;**110**: 4123–8]. Reproduced with permission of The American Society of Haematology.)

Renal disease

All patients with haemolytic PNH develop heavy renal tubular iron loading due to the continuous filtration of haemoglobin. This is evident on both magnetic resonance imaging (MRI) and post-mortem studies. The majority of patients will eventually develop chronic renal disease, with a minority progressing to established chronic renal failure requiring dialysis. In addition, during times of very intense IVH and haemoglobinuria, patients can rarely develop acute renal failure, which is potentially fully reversible, even though it may require dialysis. Some patients may develop repeated episodes of acute renal dysfunction. The use of nephrotoxic therapies, such as ciclosporin, adds to the renal insult, as do rare complications such as renal vein thrombosis.

Leukaemic transformation

There have been a number of case reports of myelodysplastic syndrome and acute myeloid leukaemia (AML) in patients with PNH, but only less than 5% of PNH patients in the larger series develop AML. This is similar to the incidence of AML in AA and since it seems the majority, if not all, patients with PNH have an underlying aplasia, then the rate of AML is no higher than would be expected, suggesting that the GPI deficiency has no impact on leukaemogenesis.

Spontaneous remission

PNH often develops in young adults between 20 and 40 years old. Historically, most patients with PNH have either died as a direct or indirect result of their disease or have suffered from the disease for the remainder of their life. In a series of 80 haemolytic PNH patients reported in 1995, initially diagnosed between 1940 and 1970, median survival was 10 years and 12 of the 35

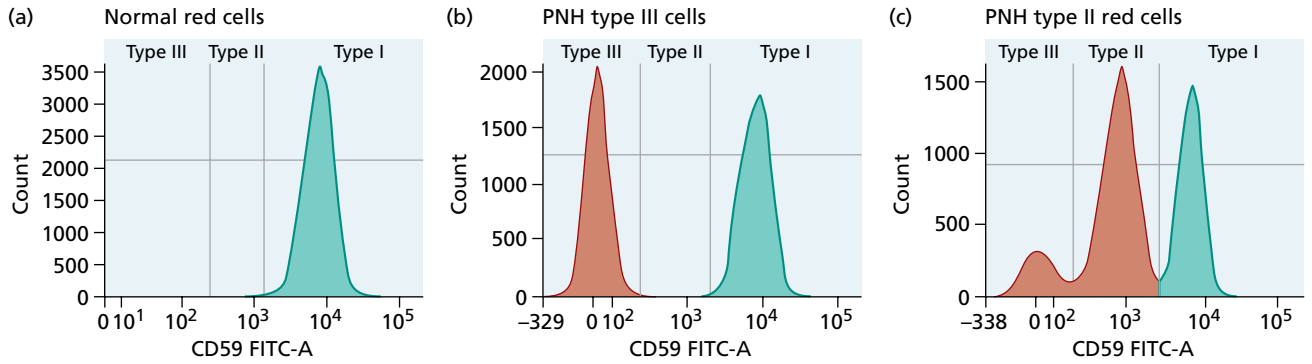


Figure 31.5 Identification of PNH red cells by flow cytometry: (a) GPI anchor protein (e.g. CD59) expression on normal red cells (type I). (b) A total of 46% of red cells are completely GPI anchor-deficient (type III). (c) a mixture of all three types of red cells are shown: type III, 10%; type II (partial GPI anchor expression), 54%; normal type I cells, 36% (Source: S.J. Richards. Reproduced with permission.)

patients surviving at least 10 years experienced spontaneous remission. Analysis of GPI-linked antigens on blood cells up to 20 years after spontaneous clinical remission demonstrated that the myeloid series was entirely normal with no residual PNH cells, whereas there was a small PNH clone in the lymphoid compartment (presumably these are long-lived memory cells). Early reports suggest that a proportion of patients treated with eculizumab have a progressive decrease in the size of their PNH clone, perhaps suggesting that remission may occur.

Investigation

The diagnosis of PNH was historically made by demonstrating the sensitivity of red cells to lysis by activated complement in the Ham test or similar. However, these tests only gave an indirect assessment of the proportion of PNH red cells and have been superseded by flow cytometry. Peripheral blood flow cytometric analysis requires analysis of at least two cell lineages (e.g. neutrophils and red cells), with a transmembrane marker to positively identify the cell type and at least two separate GPI-linked antigens to clearly separate PNH cells from their normal counterparts (Figures 31.5 and 31.6). In most patients, there is a population of red cells with complete deficiency of GPI-linked molecules (PNH type III cells, the most complement-sensitive cells) and, in some, an additional population with partial GPI deficiency (PNH type II cells, intermediate complement sensitivity). Type II and III are usually not evident in the neutrophils. The size of the PNH clone correlates with the risk of complications, such as thrombosis, and the severity of haemolysis. In addition, the evolution of the clone (either its expansion, leading to more haemolytic disease, or its reduction, ultimately leading to spontaneous remission) can be tracked.

Treatment

Supportive care

Conventional management in PNH has been supportive. The severity of haemolysis varies greatly between patients, in part dependent on the size of the PNH clone and the degree of underlying bone marrow failure. However, even patients with no evidence of clinically apparent marrow failure and large PNH clones can have highly variable levels of haemolysis. Some patients experience recurrent, even continuous, haemoglobinuria and are transfusion dependent, whereas others experience haemoglobinuria only rarely, or not at all, do not require any transfusions and have a well-compensated haemolytic anaemia.

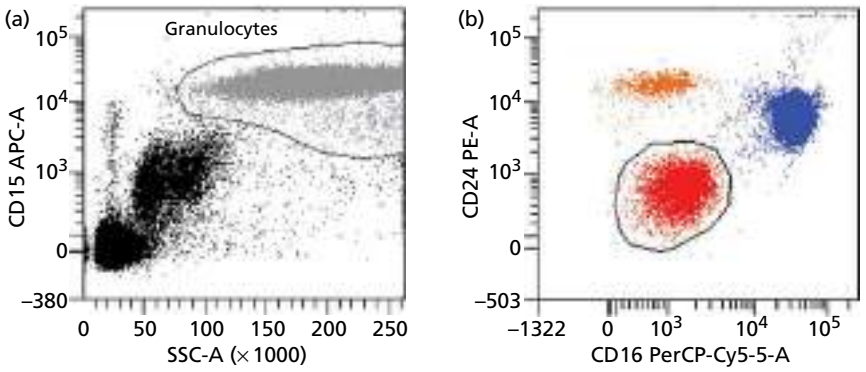


Figure 31.6 Identification of PNH granulocytes by multicolour flow cytometry: (a) granulocytes identified based on CD15 expression and side scatter. (b) analysis of CD16 and CD24 (both GPI-linked proteins) reveals a small population of PNH granulocytes (10%) that are deficient in both proteins (Source: S.J. Richards. Reproduced with permission.)

Patients with a significant degree of haemolysis should receive folic acid supplementation. The constant haemosiderinuria means that patients, even those who require regular transfusions, have a tendency to become iron deficient. This iron deficiency can result in failure of the marrow to compensate for the increased red cell destruction and therefore increasing anaemia. Iron supplementation has been reported to precipitate episodes of IVH, but usually patients tolerate oral iron supplements well and should be treated when iron deficiency is present.

Patients should be transfused according to their symptoms. Incidental infections lead to an increase in haemolysis, presumably due to activation of the complement system, and frequent sudden drops in the level of haemoglobin. These episodes are often associated with severe symptoms of abdominal pain, dysphagia and debilitating lethargy.

Corticosteroids have been widely used in the treatment of haemolysis in PNH. At high doses, steroids appear to have an effect of reducing the activity of complement and some patients report an improvement in symptoms. However, high doses are required to have a clinically useful effect and as the haemolysis in PNH is chronic, the required dose is too high. Steroids are therefore not generally recommended in PNH as the long-term side-effects outweigh the potential benefits.

Thrombosis

The major cause of morbidity and mortality in PNH is thrombosis, which usually affects the venous system, particularly the intra-abdominal and cerebral vessels, but also appears to result in arterial thrombosis (Table 31.4). The chance of developing a thrombosis depends on the size of the PNH clone and the severity of haemolysis, two variables that are closely related; half of patients with over 50% PNH neutrophils will develop a thrombosis at some point in their disease course. In most series, approximately one-third of patients will eventually die as a result of thrombosis. It appears that primary prophylaxis with warfarin is effective in preventing thrombosis, but carries a significant risk in this patient group. Aspirin does not appear to be protective against thrombosis and has no effect on the symptoms of PNH such as abdominal pain. It seems very likely that primary prophylaxis with warfarin is unnecessary for patients receiving the anticomplement monoclonal antibody eculizumab, as this agent significantly reduces the risk of thrombosis in PNH (see further on). Although there are no published data for the use of novel anticoagulants in PNH, these agents are increasingly used in clinical practice.

The treatment of established thrombosis is similar to the management of thrombosis in patients without PNH, except for the addition of immediate treatment with eculizumab (see further on). However, in view of the risk of recurrent thrombosis, patients should remain on lifelong anticoagulation after their first episode of thrombosis. Hepatic vein thrombosis (Budd–Chiari syndrome) is one of the more common thromboses seen in PNH and there have been reports of the successful lysis of such thromboses using tissue plasminogen activator; this should be considered even in patients who present with a relatively long history suggesting that their thrombosis occurred days or even weeks before. Current practice involves immediate use of eculizumab along with anticoagulation in acute Budd–Chiari syndrome, associated with PNH, to reduce mortality and long-term sequelae.

Allogeneic stem cell transplantation

The only curative strategy for PNH is allogeneic stem cell transplantation, but this carries a considerable risk of mortality. From the reported series and in view of the fact that a proportion of patients will eventually experience a spontaneous remission of PNH and with the advent of potentially effective novel therapies such as eculizumab, transplantation should only be considered in selected cases, such as those with a syngeneic donor or with associated bone marrow failure. In these patients, the indications for transplantation are similar to those for AA.

Complement blockade

The development of eculizumab (Soliris), a humanised monoclonal antibody that blocks the activation of terminal complement, has dramatically altered the management and prognosis in PNH. Eculizumab binds to C5 and stops it from being cleaved and, as long as trough plasma levels remain adequate, prevents any activation of terminal complement. Individuals with inherited terminal complement deficiency are either asymptomatic or present with recurrent *Neisseria meningitidis* (meningococcus) infections and this highlights the main concern with eculizumab. The clinical features of PNH are due largely to the absence of CD59 from haemopoietic cells and therefore the uncontrolled activity of terminal complement on PNH cells, making eculizumab an ideal candidate for the targeted therapy of PNH. In the initial study, 11 patients with transfusion-dependent haemolytic PNH were treated with eculizumab using a schedule designed to maintain trough levels to ensure that complement was completely blocked. The responses were dramatic, with an immediate resolution of the symptoms of IVH. This study was followed by a randomised placebo-controlled Phase III trial (TRIUMPH) and a non-randomised trial (SHEPHERD). In total, 195 patients were included in these three trials, which led to the licensing of eculizumab in the USA and Europe in 2007.

Ravulizumab (ALXN1210, Ultomiris), long-acting C5 inhibitor (given every 8 weekly) and modified from eculizumab (4 amino acid substituition), was shown to be non-inferior to eculizumab in 2 large pivotal phase 3 studies and led to its subsequent approval. Although C3-mediated extravascular haemolysis is likely to be similar as for eculizumab-treated patients, the 8 weekly administration is a major benefit for PNH

patients from convenience perspective in view of less frequent administration (6 infusions/year compared with 26 infusions/year for eculizumab)

Pegcetacoplan (C3 inhibitor, APL-2, Empaveli/Aspaveli) has been recently approved for treatment of PNH and clinical trial has shown superiority to eculizumab in patients with residual anaemia due to extravascular haemolysis.

Efficacy of eculizumab

Intravascular haemolysis

Eculizumab has a profound and immediate effect on IVH in PNH. The lactate dehydrogenase (LDH) concentration, which is typically as much as 10–20 times normal in PNH, falls immediately in almost all patients treated with eculizumab to normal or just above normal. In general, this results in increasing haemoglobin level which, depending on the degree of coexistent bone marrow failure as well as the extent of extravascular haemolysis (see below), will reach a plateau usually between 90 and 120 g/L. The most dramatic effect of eculizumab is on the symptoms, with resolution of the abdominal pain and dysphagia, and improvement in the severe lethargy and the other features of haemolysis. In the vast majority of patients, transfusion requirements improve, with over half of patients becoming transfusion-independent. The dramatic improvement in PNH-related symptoms is due to a marked reduction in NO consumption during therapy as a result of the improvement in IVH and thereby the reduction in free haemoglobin. This probably explains many of these benefits of the drug and provides insights into the pathophysiology of the symptoms of PNH. There is a dramatic and clinically significant improvement in the quality of life of patients as measured by validated questionnaires. There is now evidence that the adverse consequences of IVH, such as renal damage and pulmonary hypertension, are ameliorated by eculizumab.

Thrombosis

Eculizumab also protects patients from thrombosis. Compared with thrombosis before patients commencing eculizumab (effectively using patients as their own controls), the thrombotic rate reduces by fivefold to tenfold. In patients who have had a previous thrombosis and who are on anticoagulation, there is still a high recurrent thrombosis rate prior to starting eculizumab. However, since eculizumab became available, such recurrent thromboses are extremely uncommon. Patients who commence eculizumab during a 'spiral' of thrombotic events stop having further thromboses, indicating that eculizumab specifically targets the mechanism of thrombosis in PNH and that this is a more effective strategy than conventional anticoagulation alone. There is now increasing confidence that the catastrophic thrombotic complications of PNH can be much more successfully managed with a combination of eculizumab and anticoagulation. This observation will have an impact on the decision to use warfarin prophylaxis: in cases where there are concerns over the safety or requirement for warfarin prophylaxis, such as those with low platelets or with borderline PNH clone sizes, it is clearly safer to withhold anticoagulation as long as the use of eculizumab is an option for the patient should a thrombosis occur.

Renal dysfunction

In the 195 patients entering the eculizumab trials, renal dysfunction or damage was observed in 65% of patients before they were treated with eculizumab. In this series, 27% of patients had developed major clinical kidney disease within 10 years from their initial diagnosis of PNH and 21% of patients developed late-stage chronic kidney disease (stage 3 or 4 as defined by the Kidney Disease Outcomes Quality Initiative) or kidney failure (stage 5). The early analysis of patients treated with eculizumab suggests that many of the patients with early renal dysfunction (stages 1 and 2) will improve, and the deterioration in renal function in patients with advanced renal dysfunction (stages 3–5) is frequently stabilised. Thus eculizumab appears to have a beneficial effect on renal function in PNH and this is presumably due to the marked reduction in IVH and therefore in haemoglobinaemia and haemoglobinuria.

Eculizumab administration and dosing

The aim of treatment is to rapidly block complement and to maintain complement blockade continuously. The standard dosage schedule for eculizumab comprises a loading dose of 600 mg every week for 4 weeks, followed by 900 mg the next week and then 900 mg every 2 weeks indefinitely. In the majority of patients, this is adequate to maintain trough levels of eculizumab above 50 mg/L and therefore to block complement completely. In approximately 10–20% of patients, this dose is inadequate and patients break through complement blockade. Patients appear well with no signs of haemolysis until immediately before a dose of eculizumab, but then develop dark urine often with abdominal pain, sometimes with dysphagia, and a sudden deterioration in the laboratory measures of haemolysis, such as LDH and bilirubin, and a fall in the level of haemoglobin. In this situation, the maintenance dose of eculizumab is too low and an increase, either by reducing the interval or more conveniently by increasing the dose (from 900 mg every 2 weeks to 1200 mg every 2 weeks or even higher doses are usually effective), will raise trough levels of eculizumab above 50 mg/L and reestablish continuous control of haemolysis (Figures 31.7 and 31.8). This pharmacokinetic mediated breakthrough haemolysis (BTH) is not seen with long-acting C5 inhibitor ravulizumab, athough pharmacodynamic BTH due to complement amplifying conditions (CACs), like

Days		0*	1–8	9	10	11	12	13	0*	1	2
Urine scale	1 = Clear; 10 = Black	10	2–3	3	3	3	3	9	10	3	3
LDH	160–480 IU/L	2624	–	784	–	–	697	1687	2917	–	–
AST	0–40 IU/L	119	–	38	–	–	31	87	–	–	–
PK	C5 blocked >35 μg/mL	27	–	45	–	–	35	29	26	–	–
PD	No lysis <20%	71	–	1	–	–	2	56	56	–	–

*Eculizumab 900 mg

Figure 31.7 Breakthrough from complement blockade by eculizumab due to inadequate dose level. The urine is clear with lactate dehydrogenase (LDH) just above normal and no haemolytic activity in the patient's serum at day 12 after a 900-mg dose of eculizumab. On day 13, the patient suddenly begins to haemolyse as the level of eculizumab falls below 35 mg/L (the level at which complement is blocked). Immediately after the next dose (day 0), the haemolysis stops. Urine scale: patient assesses urine colour first thing in the morning (red or black urine at 6+). AST, aspartate aminotransferase; PD, pharmacodynamics (haemolytic potential of the patient's serum *in vitro*); PK, pharmacokinetics (serum level of eculizumab).

surgery, infection is seen with any pharmacological complement blockade and is less well understood.

Approximately 3% of Japanese individuals have a C5 polymorphism that prevents the binding of eculizumab/ravulizumab and means that they do not respond with no fall in their LDH after dosing.

Extravascular haemolysis (EVH) with eculizumab

Despite the impressive impact on IVH, most patients remain somewhat anaemic, maintaining their haemoglobin between 90 and 120 g/L. Virtually all have a persisting reticulocytosis and

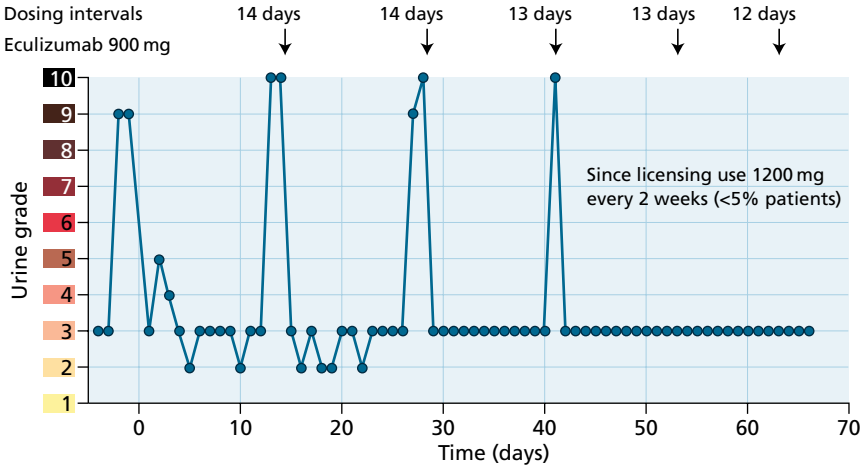


Figure 31.8 Urine colour with increasing eculizumab dose. Patient records early-morning urine colour according to scale on *y*-axis. He is breaking through from complement blockade for 2 days when receiving 900 mg eculizumab every 14 days. When the frequency of eculizumab 900 mg is increased to every 12 days, his breakthroughs cease. He is now maintained on 1200 mg eculizumab every 14 days.

many continue to have a raised bilirubin. These features are suggestive of ongoing extravascular haemolysis, which has previously been unreported in PNH. On further investigation, it transpires that approximately two-thirds of PNH patients on eculizumab develop a positive direct antiglobulin test to complement only. Flow cytometry demonstrates that the PNH red cells are coated by early complement components (C3b and C3d), possibly because PNH red cells, as well as being deficient in the principal controller of terminal complement, namely CD59, do not express the inhibitor of C3 convertase decay-accelerating factor (DAF). It appears that preventing terminal complement activation leads to a build-up of the early complement components, which accumulate on PNH red cells due to their deficiency of DAF. If intense, this extravascular haemolysis results in a poor increase in haemoglobin, and a minority of patients continuing to require transfusions. The recently approved C3 inhibitor pegcetacoplan has shown remarkable benefit in patients with residual anaemia due to EVH. The phase 3 trial compared eculizumab with pegcetacoplan and showed superiority via broad haemolysis control and hence improving haemoglobin and abolishing transfusion independency in 85% of patients. The drug is self-administed by subcutaneous infusion twice weekly and is fairly well tolerated.

Infectious risk with complement blockade

Eculizumab is generally very well tolerated with few infusion-related reactions. However, as noted above, blocking terminal complement activity would be expected to increase the risk of infection with *Neisseria meningitidis* (meningococcus). All patients commencing eculizumab should be vaccinated with a wide-spectrum meningococcal vaccine (ACWY Vax) and recently approved serotype B (Bexsero) vaccine. Although the risk of meningococcal infection is a concern, the observed risk is 0.25 cases of meningococcal infection per 100 patient years on eculizumab. However, when these infections occur they can be life-threatening and it is vitally important to impress on patients that they should seek medical help if they suffer any symptoms suggestive of infection, and these are usually of septicaemia rather than meningitis. Prophylaxis with penicillin is also recommended. There is no convincing evidence as yet of an increased risk to any other organism except for *N. meningitidis*. Patients on C3 inhibitors are theoretically at increased risk of infections with other encapsulated organisms and hence are additionally vaccinated against S.pneumonia and H.influenza.

Pregnancy in PNH

It is difficult to estimate the true risk of pregnancy in PNH but there is undoubtedly an increased risk of maternal morbidity and mortality. The reported maternal mortality, mainly from thrombosis, is between 12 and 21%, although this is likely an overestimate due to biassed reporting. In addition, an increased fetal loss rate has been reported, although again this is difficult to substantiate and is probably due to maternal factors. There have now been a number of successful pregnancies in women receiving eculizumab either later in pregnancy or throughout the pregnancy from conception to delivery. The reports to date are positive with little or no eculizumab crossing the placenta into the fetus. It does appear that the metabolism of eculizumab may be altered in pregnancy and women seem more likely to break through complement blockade and may need higher doses in the latter part of pregnancy. No safety data is available for ravulizumab and pegcetacoplan in pregnancy.

Prognosis

A series of 80 patients with haemolytic PNH diagnosed between 1940 and 1970 were reported in 1995 to reveal a median survival of approximately 10 years from initial diagnosis of PNH. A more recent series of 465 patients reported from France, including patients with small PNH clones as well as haemolytic PNH, reported a median survival of 22 years. The major causes of death in haemolytic PNH are thrombotic and since these are virtually abolished by eculizumab/ravulizumab therapy, and multiple reports have demonstrated that the survival of patients with PNH receiving eculizumab is very similar to an age-matched control population.

Future challenges and developments

The development of targeted therapy with C5 inhibitors (eculizumab/ravulizumab) and C3 inhibitors (pegcetacoplan) for PNH appears to alter the natural history of the disease and prevent morbidity and mortality. Newer anticomplement therapies, especially oral agents targeting specific proximal pathways like Factor D and Factor B are being evaluated in advanced Phase 3 trials. A global PNH registry has been established to document the changes in the natural history of the disease, as well as to record any unexpected complications of the newer therapeutic interventions. The current therapy of PNH controls rather than cures the disease. It is hoped that with more detailed understanding of the pathophysiology of PNH, a curative strategy may be developed.

Suggested further reading

Clinical significance of acquired somatic mutations in AA

The group at King's College Hospital, London, reported the first large study of acquired somatic mutations in AA patients using next-generation sequencing. Almost 20% of patients had mutations in myeloid-specific genes, most commonly *ASXL1, DNMT3A* and *BCOR*. Subsequently, a larger study from NIH,

Cleveland Clinic USA and Japan has confirmed similar results with *ASXL1, DNMT3A, BCOR* and *BCORL1* in 36% of AA patients. In both studies, the size of these mutated clones in AA was small in most patients and lower than found in MDS. The presence of somatic mutations was highly predictive of later evolution to MDS/AML (in 40% patients) compared to only 4% who lacked these mutations, for patients with a history of AA of >6 months. Mutations in *ASXL1* and *DNMT3A* were associated with worse response to immunosuppressive therapy and worse survival; in contrast, *BCOR/BCORL1* and *PIG-A* mutations conferred better response to IST and better survival. When all these mutations are combined with single nucleotide polymorphism (SNP-A) karyotyping, evidence of clonal haemopoiesis was demonstrated in 47% of AA patients. However, the interpretation of clonal haemopoiesis in AA is not straightforward. Age-related clonal haemopoiesis with mutations in especially *ASXL1* and *DNMT3A*, and others including *TET2, JAK2* but not *BCOR/BCORL1* or *PIG-A*, has been reported in 10% normal individuals older than 65 years, and increasing further with subsequent decades of life. In AA, analogous to PNH and abnormal cytogenetic clones, the level of these clones may fluctuate over time and may be selected in the context of immune dysregulation in the setting of bone marrow failure through increased proliferative pressure on the residual haemopoietic stem cell/progenitors. A possible unifying explanation is that the low-level 'age-related' clones represent a predilection (founder) stage that requires subsequent cooperating mutations for clonal expansion and disease, alongside the increased telomere attrition seen in AA. The RACE trial (below) showed that nearly 30% of SAA patients had somatic mutations with VAF >1% at diagnosis and this increased to up to 66% at 6 months and did not adversely impact haematological responses or 2-year outcome, including clonal evolution. This indicated the ubiquitous nature of clonal haematopoiesis in AA and the caution needed by physicians in overinterpreting somatic mutations in isolation, especially in treatment decisions.

Eltrombopag added to IST in first-line treatment of AA

The utility of Eltrombopag in inducing haematological responses in 40% of multiply pre-treated refractory AA was demonstrated in 2012, and subsequently led to its evaluation in first-line setting to utilise its HSPCs stimulatory properties. A single-arm NIH study showed its utility in the upfront setting when used in combination with conventional IST. The RACE trial, an international, investigator-driven, open-label, phase III, randomised trial evaluated 197 patients with acquired SAA (aged 15 years or older) who had not received prior immunosuppressive treatment. Patients were randomised to receive either standard immunosuppression (horse ATG 40 mg/kg × 4 days and CSA 5 mg/kg/day) or standard immunosuppression + eltrombopag at the dose of 150 mg/day from day +14 until 6 months. The primary endpoint of the study was complete response (CR) at 3 months- defined as haemoglobin 10 g/dL, neutrophil count $>1 \times 10^9$/L and platelet count $>100 \times 10^9$/L. Patients who received the combination of hATG, CSA plus eltrombopag had a significantly higher complete response rate at 3 months compared to patients treated with horse ATG and CSA alone (22 versus 10%). These higher response rates were sustained at 6 months (overall response of 41% with standard treatment versus 68% with standard treatment + Eltrombopag). The median time to first response was 9 months with standard treatment versus 3 months with standard treatment + eltrombopag. In addition, eltrombopag was generally well-tolerated, with a similar occurrence of adverse events in the two treatment arms. Although triple-drug therapy is the standard of care in USA for treatment naïve SAA, regulatory approval would facilitate its use in Europe and other countries.

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CHAPTER 32

Acute myeloid leukaemia

32

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Epidemiology and demographics

Acute myeloid leukaemia (AML) is the commonest acute leukaemia in adults and has the shortest survival. AML has an incidence of around 5 cases per 100,000 population per year and is ~1.4 times more common in males. Age is by far the most significant risk factor for developing AML; AML incidence increases from 1/100,000 per annum in children and then peaks at around 28/100,000 cases per annum in 80–84-year-olds (Figure 32.1). Aromatic hydrocarbons, such as benzene are currently the only proven occupational or lifestyle factor that causes AML, although associations with smoking, obesity and fungicide exposure have also been reported. Therapy-related AML (tAML) as a result of chemoradiotherapy or immunotherapy for another malignancy is well described and, due to the increased use of adjuvant chemotherapy for breast and colon cancer, its incidence is on the rise. Clonal haematopoiesis of indeterminate potential (CHIP) (Chapter 38), is the phenomenon of expansion of clones containing somatic mutations in individuals with no overt evidence of haematological disease. Although a risk factor for AML, where its presence may precede disease development by many decades, progression is not an inevitable outcome, and factors that facilitate progression are yet to be determined.

Due to improvements in induction and consolidation therapy, stem cell transplant (SCT) delivery and supportive care, AML survival has improved dramatically for younger patients. In patients aged between 15 and 39 years, 5-year survival increased from 41% in 1990–1999 to 63% in 2010–2017 in the USA. However, recent 5-year survival (2010–2017) in patients >70 remained dismal at only 5%, with these figures not only reflecting both patient co-morbidities and limited treatment options, but also worse disease biology. Despite this, expanding adoption of more intensive treatments for older patients, improved supportive care and recent developments of less toxic targeted-AML therapies are finally starting to improve outcomes in older AML patients.

Clinical presentation

AML is characterised by an overproduction of immature myeloid blasts at the expense of mature granulocytes, lymphocytes, red cells and platelets. Fatigue, anorexia, weight loss and night sweats are common constitutional symptoms in people with AML, while extensive marrow infiltration can occasionally manifest in bone pain. The consequent bone marrow failure may cause symptoms of anaemia (fatigue, shortness of breath, light-headedness, pallor, headaches or feeling cold), thrombocytopenia (easy or spontaneous bruising, petechiae, bleeding gums, epistaxis and heavy periods) and neutropenia (susceptibility to bacterial, fungal, viral or protozoal infections). White cell counts (WCC) vary significantly and around 10% of AML patients present with hyperleucocytosis (WCC >100 × 10^9/L).

Hepatomegaly, splenomegaly and lymphadenopathy can occur but are generally uncommon in AML. Gum infiltration and hypertrophy are common in AML that has monocytic or myelomonocytic differentiation and *Leukaemia cutis* – cutaneous infiltration by leukaemic cells – is seen in 10–15% of cases, and up to 50% of cases of acute

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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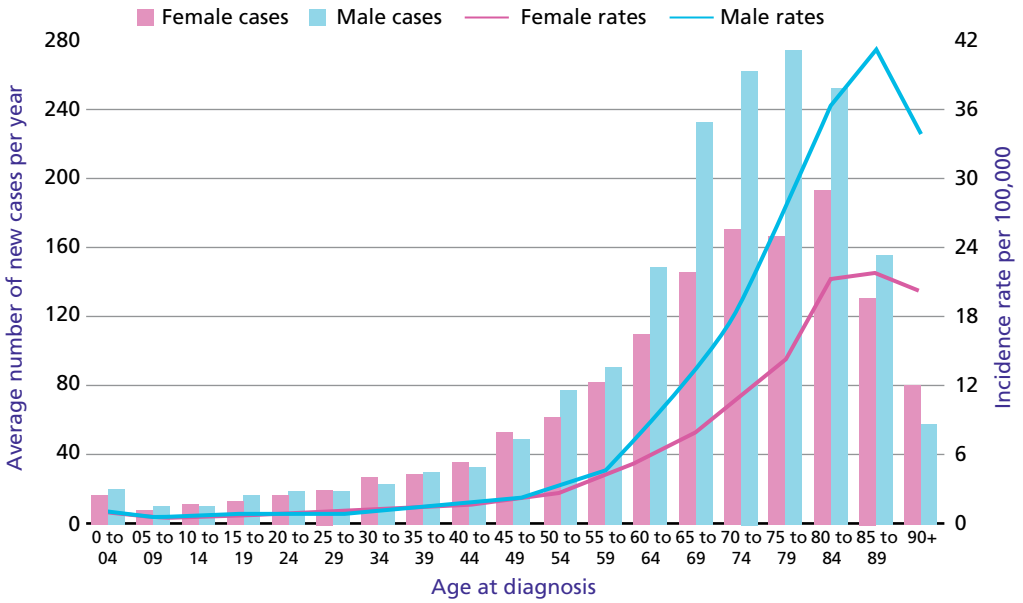

Figure 32.1 Incidence of AML per age group and sex in UK in 2017 (Source: Data from Cancer Research UK.)

myelomonocytic leukaemia. Central nervous system infiltration is a rare manifestation of AML (<1% of cases) with poor prognosis and is more frequent at relapse (~3% of cases). AML can occasionally present with extramedullary masses – termed myeloid sarcoma or chloroma. The t(8;21)/*RUNX1::RUNX1T* translocation is the commonest cytogenetic abnormality associated with myeloid sarcoma, although inv(16)/*CBFB::MYH11* also demonstrates an increased incidence.

Specific problems may include: leucostasis, tumour lysis syndrome and disseminated intravascular coagulation (DIC). In contrast to the ~5% chance of death within the first week in patients without hyperleucocytosis, hyperleucocytosis is associated with an up to 20% risk of death, owing to thromboembolic, pulmonary and neurological complications. Leucostasis is rare but can manifest in blurred vision due to retinal bleeds, mucosal bleeding and neurological symptoms ranging from vertigo, headaches to stroke-like symptoms, seizures and coma in the most severe cases. Clinical or laboratory tumour lysis syndrome is seen in around 15% cases with induction chemotherapy, especially if pre-therapy WCC $>25 \times 10^{\wedge}9$/L. DIC is a common presenting feature and cause of death in acute promyelocytic leukaemia (APML), but its risks can be almost reversed by prompt treatment with all-trans retinoic acid (ATRA). DIC is uncommon in other non-APML subtypes of AML, but occurs in up to 40% of AML with hyperleucocytosis, where it should be managed with platelet and coagulation factor support.

Molecular pathogenesis and genetic abnormalities

AML is characterised by recurrent genetic abnormalities at the chromosome and single-gene level. These heterogeneous lesions cooperate to generate the uniform phenotype of AML: clonal expansion, differentiation block and increased survival of myeloid progenitors. AML can develop secondary to antecedent haematological malignancies such as myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN) (secondary AML, sAML, ~10–20% of all AML cases) or following treatment for another cancer (particularly alkylating agents or topoisomerase II inhibitors) (tAML ~10–15% of cases). However, most AML cases develop sporadically (*de novo* AML), although the recently described process of clonal haematopoiesis (CH) (Chapter 38) suggests that, in many, this is the culmination of many decades of clonal growth and progression. Initial insight into genetic anomalies in AML came from the characterisation of recurrent balanced chromosomal rearrangements, loss or gain of chromosomal material or of entire chromosomes/chromosome arms. The advent of massively parallel genomic sequencing techniques has propelled the understanding of the molecular landscape of AML through identification of multiple driver mutations and members of different biological pathways that underlie AML development. AML was the first cancer to be sequenced and due to the ease of obtaining tumour tissue, remains one of the most sequenced human neoplasms. Genetic alterations in AML occur non-randomly: certain genetic aberrations sequentially co-occur or are mutually exclusive, suggesting that AML develops following the engagement/disablement of set pathways. The observation of CH suggests that, in many cases, this process occurs over many decades.

Cytogenetic abnormalities

Balanced chromosomal rearrangements underlie AML pathogenesis in about one-third of cases (Figure 32.2). Chromosomal

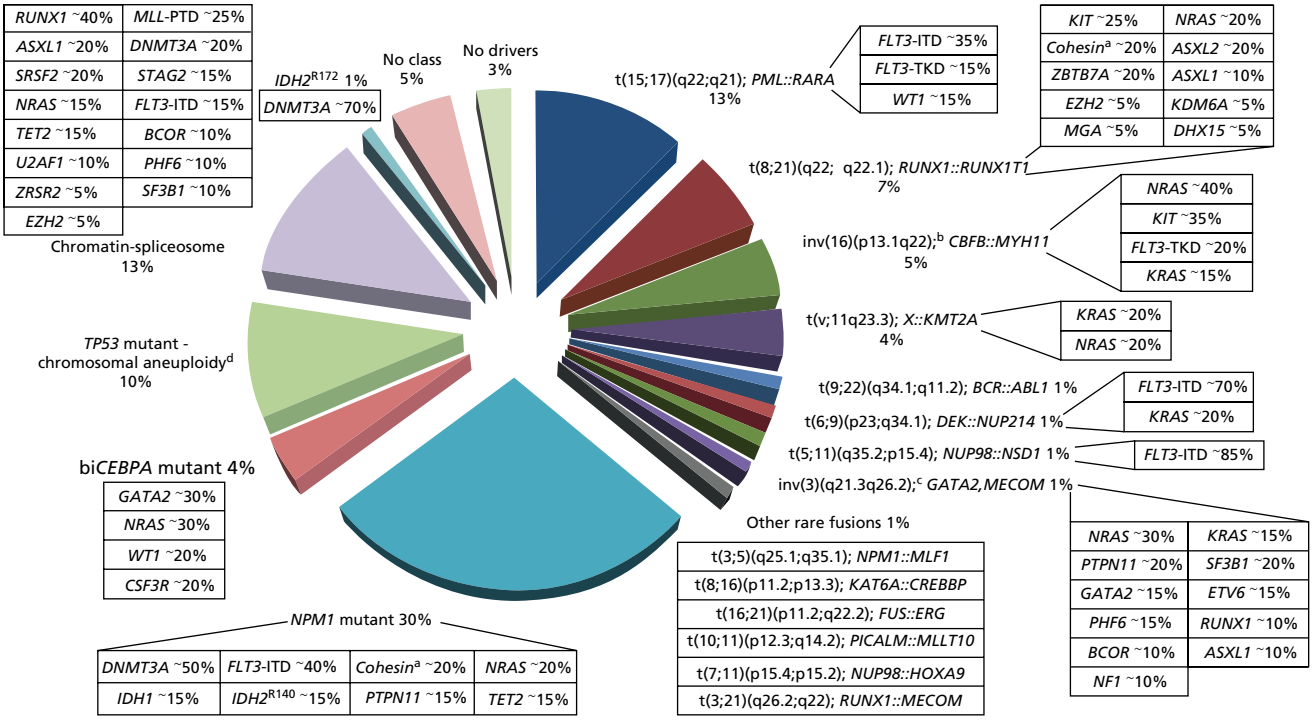

Figure 32.2 Molecular landscape of AML in younger adults (Source: Grimwade *et al.* 2016; Döhner *et al.* 2017.)

rearrangements or inversions in AML result in chimeric oncoproteins and frequently target myeloid transcription factors (for example, *RUNX1, RARA* or *CBFβ*), epigenetic regulators (*KMT2A, KMT3A* and *NSD1*) or nuclear pore complex members (*NUP98* and *NUP214*). Novel fusion proteins can exert their leukaemogenic effects through either disruption or alteration of native target protein function.

Core-binding factor leukaemia – Translocation between chromosomes 8 and 21, the t(8;21) leading to the *RUNX1::RUNX1T1* fusion and inversion of chromosome 16 (inv16), that rearranges to generate the *CBFB::MYH11* fusion, occur in ~12% of AML cases and confer favourable prognosis. Both result in the disruption of subunits of the core-binding factor complex (*RUNX1::CBFB*) necessary for the maturation of myeloid cells.

PML::RARA – *RARA* (retinoic acid receptor α) is a transcription factor regulating the expression of genes involved in myeloid differentiation. The *PML::RARA* fusion, a hallmark of acute promyelocytic leukaemia (APML), results from the t(15;17). This resultant fusion protein leads to the recruitment of transcriptional repressor proteins to RARA targets and subsequent maturation block in myeloid cells. Notably, *RARA* can have other fusion partners, which account for a minority of APML cases.

KMT2A rearrangements – *KMT2A* (also known as *MLL1*) is a chromatin methyltransferase that acts as a co-activator of gene expression in early haematopoiesis. *KMT2A* rearrangements in AML can occur with over 70 different partners, but *AF4, AF9, AF10* and *ENL* account for more than 75% of cases. *KMT2A* fusions positively enhance the function of the super-elongation complex and DOT1L methyltransferase to increase transcription at critical leukaemia genes where they require the co-factor Menin to bind.

Aneuploidies and unbalanced chromosomal abnormalities – Recurrent structural chromosome abnormalities are also frequently found in AML. Aneuploidies (loss or gain of entire chromosomes) and unbalanced chromosomal rearrangements (deletions and/or duplications) occur in around 20% of AML. Loss events are more common than gain in AML. Loss of chromosome 7 (−7) is the commonest monosomy in AML and occurs in around 5% of all AML patients, while +8 is the commonest trisomy (~10% prevalence). Loss of material from chromosome 5 long arm (5q), 7q and 17p are the commonest loss of chromosome material events. While the molecular consequences of gain of chromosomal material are less well understood, loss leads to the haploinsufficiency of tumour suppressor genes. In 17p loss, one copy of the *TP53* gene, a key tumour suppressor gene (*TSG*), is lost generating genetic instability, development of further karyotypic anomalies, and generates an AML with particularly poor risk. The situation is more complex to generalise in regions with potentially large regions of loss, such as del 7q and 5q, but this almost certainly also leads to loss of TSGs, such as *MLL3, CUX1, EZH2,* etc.

'Complex' Karyotype – The presence of more than three chromosome aberrations (excluding t(15;17), t(8;21), inv(16) and *KMT2A*-rearrangements) constitutes AML with a complex

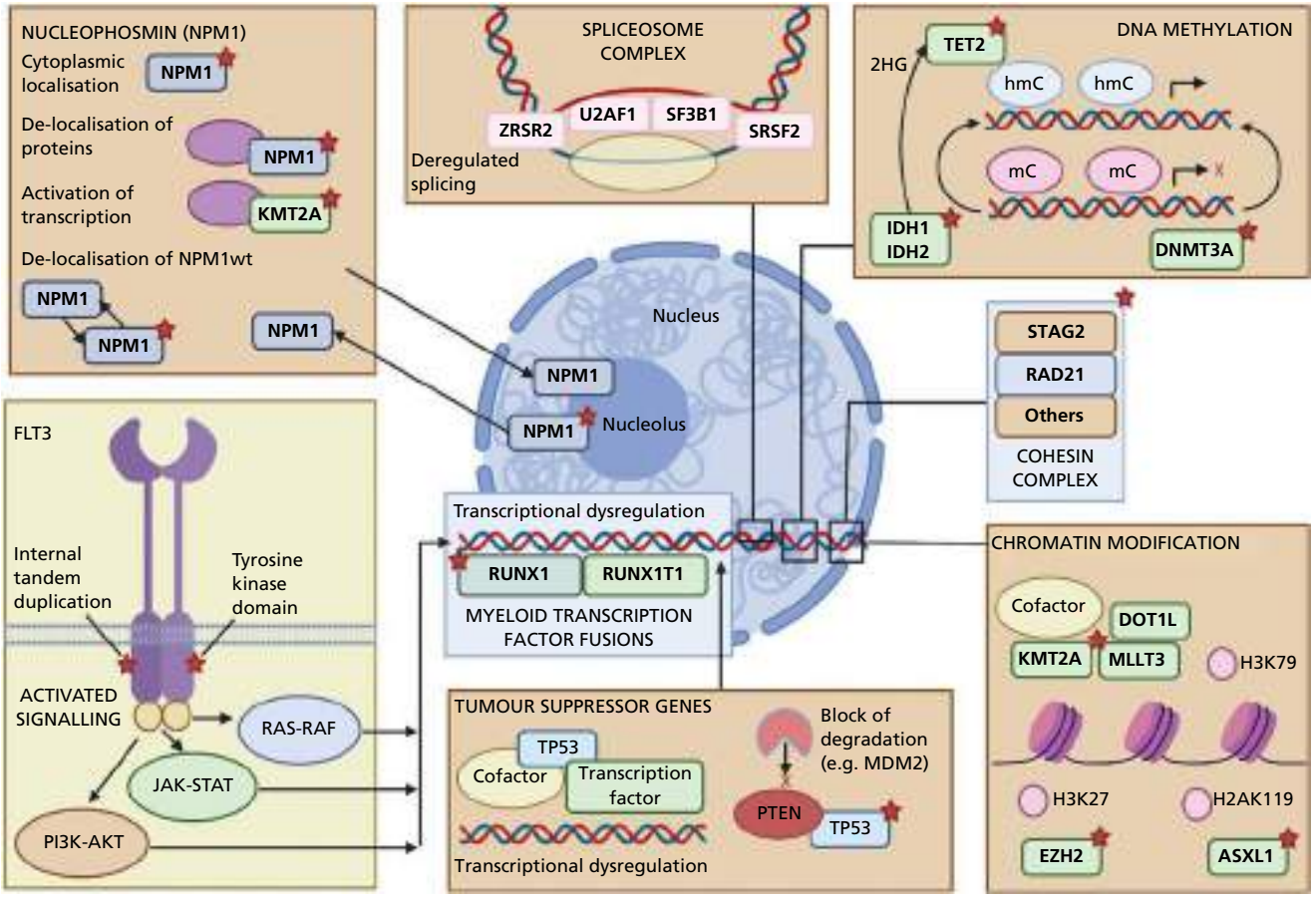

Figure 32.3 Functional classification of common driver mutations in AML (Adapted from Döhner et al, N Engl J Med, 2015, using BioRender.)

karyotype. This accounts for ~10–12% of all AML cases and its incidence dramatically increases with age. Due to its requirement for genome integrity, *TP53* is often mutated/lost in complex karyotype AML and there is a higher incidence of prior exposure to alkylating agents, topoisomerase II inhibitors and radiotherapy. Complex karyotype AML is associated with a very poor prognosis.

Somatic point mutations

More than 100 driver genes have been identified in AML. As with chromosomal rearrangements, isolated mutation can provide Haematopoietic stem and progenitor cell (HSPC) clones with a proliferative advantage, however on their own they are insufficient to induce leukaemia. With currently used DNA sequencing panels, driver mutations are identified in at least 96% of AML cases. Two or more driver mutations are detected in 86% of AML patients and AML patients carry an average of between three and five molecular lesions. The pattern of co-mutation in AML is not random: mutations co-occur at frequencies higher than would be expected by chance and can also be mutually exclusive, suggesting cooperative activation of specific core downstream developmental pathways in AML. Mutations in key AML driver genes can be divided in 8 functional categories (Figure 32.3).

Nucleophosmin 1 (~25% cases)- Nucleophosmin 1 (NPM1) is a nucleolar protein involved in mRNA transport, ribosome synthesis, chromatin remodelling, apoptosis and genome stability. *NPM1* mutations are one of the most common genetic aberrations in AML but have not been detected in any human cancers other than AML. They appear specific for *de novo* AML, are almost always mutually exclusive with chromosomal translocations or structural alterations (normal-karyotype AML) and are late events in leukaemogenesis. *NPM1* mutations cause cytoplasmic dislocation of the protein and are associated with expression of stem cell genetic programmes, such as *HOXA* and *HOXB* genes and reduced apoptosis.

Signalling cascade (collectively ~60% AML) – Mutations constitutively activating Ras-MAPK-STAT5 signalling pathway maintain AML blast proliferation, cell survival programmes and are amongst the commonest genetic alterations in AML. Fms-like tyrosine kinase 3 (*FLT3*) is a receptor tyrosine kinase and is one

of the most commonly mutated genes in AML. *FLT3* internal tandem duplication (*FLT3::ITD*) and *FLT3* tyrosine kinase domain point mutations (*FLT3::TKD*) are found in 25 and 7% of AML cases, respectively. While *FLT3::ITD* is associated with adverse prognosis, *FLT3::TKD* influence on AML prognosis remains uncertain. Mutations in other signalling pathways members *cKIT, KRAS, NRAS, PTPN11* and *NF1* are also seen at lower frequency. Signalling mutations co-occur with multiple other mutations and are particularly common with chromosomal translocations. In general, they alter the risk stratification of patients that carry them, although this may be modified in the future by available therapies targeting these pathways.

Epigenetic modifiers (collectively ~80%) – Mutations in epigenetic modifiers are amongst the earliest events in AML pathogenesis and are the most commonly found mutations in CH. Mutations occur in both regulators of DNA methylation and histone regulation. *DNMT3A* is a DNA methyltransferase and is mutated in around 20% of AML cases. The mutations are heterogeneous, but the commonest (~60% of cases) occurs in codon R882, which generates a dominant-negative protein able to bind to and inactive the wild type DNMT3A. Abrogation of DNMT3A function in HSCs appears to favour self-renewal over differentiation during stress, although the mechanism that drives this is not yet clear. *TET2* is a DNA demethylase, which removes methyl groups by converting them into a hydroxymethyl group and is mutated in ~15% of AML. *TET2* mutations appear to facilitate stem-like transcriptional programmes. *ASXL1* mutations occur in ~6% of AML cases, are more common in elderly, secondary AML, and may be independent adverse prognosis risk factor due to increased resistance to chemotherapy. *ASXL1* interacts with the chromatin-modifying polycomb repressive complex 2 (PRC2) members *EZH2* and *SUZ12*, that are also mutated in AML, and the BAP1 deubiquitinase to regulate deposition of the repressive histone marks H3K27me3 and H2AK119Ub and expression of leukaemia genes such as the *HOXA* cluster genes and *IRF8*. Mutations in isocitrate dehydrogenases *IDH1* and *IDH2* (respectively ~7 and 9% of AML) generate neomorphic enzymes that further process α-ketoglutarate to 2-hydroxygluterate (2-HG), an oncometabolite that inhibits α-ketoglutarate-dependent dioxygenase enzymes such as TET2. *IDH1* and *IDH2* mutations are mutually exclusive of *TET2* mutations but can be targeted by novel specific inhibitors Ivosidenib and Enasidenib, respectively.

Spliceosome regulators – Mutations in spliceosome genes (*SRSF2, SF3B1, U2AF1* and *ZRSR2*) occur in ~15–20% of AML and are considered to be pathognomonic of AML that has arisen from MDS. The mutations are therefore early founder mutations in leukaemogenesis and alter splicing of multiple target proteins via usage of alternative cryptic 3′ splice sites (*SF3B1* mutations), exon skipping (*U2AF1*) or exon inclusion (*SRSF2*).

Myeloid transcription factors – RUNX1 and CEBPA are two pivotal transcription factors controlling haematopoiesis and differentiation, and are commonly mutated in AML, each at frequencies of up to ~10% of cases. RUNX1 is a core binding family master transcription factor that controls haematopoietic differentiation, ribosome biogenesis, cell cycle and TP53 function. Point mutations in *RUNX1* are seen across *de novo* AML, therapy-related AML and AML resulting from MDS or MPN and disrupt normal RUNX1 production and function and are associated with poor-prognosis disease. *CEPBA* mutations can occur at both N and C-domains and are associated with favourable prognosis only when they occur in-frame in the basic leucine-zipper (bZIP) region, regardless of being biallelic or monoallelic mutations. Notably, mutations of both *RUNX1* and *CEBPA* can be inherited through the germline (see below).

Tumour suppressor genes (TSG) – Archetypal TSG lesion include *TP53* mutations, that are detected in ~10% of AML cases. *TP53* mutations alter the DNA damage response to genotoxic stress and are strongly associated with tAML, complex karyotype and disease of dismal prognosis, regardless of other prognostic factors.

Cohesin complex – The multisubunit Cohesin complex is responsible for sister chromatid cohesion in mitosis, homologous recombination, DNA looping to permit gene-regulation and DNA damage response. Mutations in Cohesin complex members (*STAG2, RAD21, SMC3, SMC1A*) occur in ~10% of AML, and preliminary results suggest their mutation causes dysregulated differentiation and stem-cell programmes via altered transcriptional regulation.

Germline mutations

It is now increasingly recognised that some mutations predisposing to AML and MDS are inherited through the germline. This emphasises the clinical importance of taking a detailed personal and family history of bleeding episodes and of other haematological and non-haematological malignancies. Identification may permit genetic counselling and necessitates germline testing for family haematopoietic stem cell donors. Germline mutations predisposing to AML can also predispose to platelet disorders, organ dysfunction and other malignancies (Table 32.1). Notably, mutations found in homologous DNA recombination repair pathway and familial cancer syndromes (*BRCA1* and *BRCA2, CHEK2, TP53, PALB2* and Lynch syndrome mutations) are found in around 20% of AML cases and may prompt screening of other family members. Bone marrow failure syndromes, many of which predispose to AML, are discussed in Chapter 30.

Germline mutations in master myeloid transcription factors *RUNX1, CEBPA* and *GATA2* were the first to be implicated in familial AML. Patients with germline *RUNX1* mutation have familial platelet disorder with around 44% risk of lifetime penetrance to myeloid malignancy, usually in adulthood. Nearly 10% of AMLs with biallelic *CEBPA* mutations harbour germline mutations. Germline *CEBPA* mutations usually occur in the 5′ end of the gene and cause AML with nearly complete

Table 32.1 Commoner germline predisposition to AML.

Gene or pathway	Haematologic malignancy	Additional haematologic abnormality	Other malignancies	Additional symptoms	Syndrome
CEBPA	AML	No common abnormalities noted	No other malignancies are common	None observed	Familial AML with mutated *CEBPA*
DDX41	MDS and AML; less commonly lymphoma	Cytopenia	No other malignancies are common	None observed	Familial AML with mutated *DDX41*
GATA2	MDS and AML	Immunodeficiency, bone marrow failure, monocytopenia and B cell lymphopenia	No other malignancies are common	Lymphoedema, pulmonary alveolar proteinosis and congenital deafness	Emberger syndrome (OMIM 614038) and MonoMac syndrome (OMIM 614172)
RUNX1	MDS and AML; less commonly T-ALL	Thrombocytopenia and decreased platelet function	No other malignancies are common	None observed	Familial platelet disorder with propensity for myeloid malignancy (OMIM 601399)
ETV6	ALL, MDS, AML	Thrombocytopenia and decreased platelet function	No other malignancies are common	None observed	Thrombocytopenia 5 (OMIM 616216)
ANKRD26	AML	Lifelong thrombocytopenia and various platelet dysfunctions	Not described	None observed	Thrombocytopenia 2 (OMIM 188000)
TP53	MDS and AML	Not observed	Osteosarcoma, breast cancer, soft tissue sarcoma, brain tumours and adrenocortical carcinoma	None observed	Li Fraumeni Syndrome (OMIM 151623)
Trisomy 21	AML and ALL	Transient abnormal myelopoiesis	No other malignancies are common	Mental retardation, cardiac abnormalities and facial dysmorphologies	Down syndrome (OMIM 190685)

(*Source:* Adapted from Klco J, Mullighan CG *Nat. Rev. Cancer* 2021 https://www.nature.com/articles/s41568-020-00315-z/tables/1.)

penetrance in young adulthood, but generally have a good prognosis. Along with many other syndromes, germline *GATA2* mutations predispose to MDS and AML with at least 33% lifetime risk. Germline mutations in the RNA helicase *DDX41*, cause MDS and AML with long latency but *DDX41 R525H* mutation also commonly arises somatically. Children with Down's syndrome have 150-times higher risk of AML in the first 5 years of life. Myeloid leukaemia associated with Down syndrome is a separate classification in both ICC 2022 and WHO 2022 and is predominantly magakaryoblastic in morphology. Transient abnormal myelopoiesis develops within the first 6 months of life in 30% of children with trisomy 21 (which can be mosaic), invariably accompanied by mutation in exons 2 or 3 of *GATA1*, with 20–30% progressing to AML upon acquisition of further somatic mutations. Common germline predisposition genes are shown in Table 32.1.

Clonal evolution and disease relapse

Morphological complete remission (CR) will be achieved following induction therapy in most AML patients. However, relapse eventually occurs in the majority of these patients and is the chief cause of AML mortality. Although the mechanisms are still poorly understood, leukaemic cells can acquire new genetic mutations and epigenetic modifications or rely on the microenvironment to survive, adapt and regrow following AML therapy. An important substrate in this ability is intratumoural heterogeneity; multiple leukaemic subclones are known to exist in a single patient. Moreover, specific leukaemia stem or initiating cells (LSC) have been demonstrated in AML. Growing evidence suggests that relapse can occur from dominant clones or subclones evident at diagnosis or from clones that have evolved during treatment. Indeed, particularly related to the more targeted therapies available, we are developing biological insights into adaptive treatment resistance and the scientific basis for relapse at both the genetic and non-genetic levels. Finally, recent studies have also shown a significant non-cell autonomous component to the development of resistance, driven by the AML microenvironment.

Genomic clonal architecture can be computationally inferred from bulk DNA sequencing of AML using variant allele frequencies (VAF). The advent of single-cell DNA sequencing and its application to longitudinal samples from the same patient has further allowed greater resolution of clonal evolution and the genetic lesions driving this. For example, resistance that develops to AML patients treated with FLT3 inhibitors is often accompanied by the expansion of clones that either demonstrate a resistant *FLT3* F691L mutation or mutation of downstream MAPK-RAS signalling pathway intermediates (*NRAS* and *KRAS*), which both restore downstream FLT3 signalling. Similar mutations that prevent binding of inhibitors to mutant IDH1 and 2 have also been described, as has the phenomenon of 'isoform switching'. In this scenario, an *IDH1*-mutated AML patient treated with the IDH1 inhibitor Ivosedenib develops an *IDH2* mutation to restore 2HG production and aberrant leukaemic transcriptional programmes, or vice-versa for an *IDH2*-mutated patient.

Clonal evolution can be linear, branched or simultaneously exploit both patterns (Figure 32.4). Clones responsible for disease relapse often pre-exist at low levels and are selected for by treatment or arise early in treatment (for example *NRAS*-mutated clones). Genetic clonal evolution seems to be an early event upon treatment initiation, before overt disease relapse and may provide a window for intervention in the future. However, often no new detectable genetic abnormalities are detected at AML relapse and mutations cannot alone explain disease refractoriness. Therefore, investigations of non-genetic mechanisms of resistance, including the role of the microenvironment, are urgently required.

Disease classification

Over the past decade, AML has undergone a radical shift from morphological and phenotypic classification to a primarily genetic classification of the disease. There are currently two independent, but greatly overlapping, classification systems for AML: the International Consensus Classification (ICC) and the World Health organisation (WHO) systems, both of which were updated in 2022. Given its extensive integration with prognostication and therapeutic frameworks, the ICC AML classification will be used in this chapter.

Recurrent genetic abnormalities, if present, are the most important diagnostic attributes in the hierarchical approach to AML diagnosis taking precedence over purely karyotypic or phenotypic criterion (Table 32.2). More than 10% myeloid blast count in PB or BM is sufficient to establish a diagnosis of AML in the presence of a recurrent genetic abnormality. Recognition that high blast count MDS patients often derive benefit from AML therapies, has led to the softening of the boundary between AML and MDS. As a result, cases with 10–19% myeloid blasts in BM or PB without recurrent AML-defining genetic abnormalities are classified as MDS/AML. Owing to its particularly poor prognosis, AML with mutated *TP53* constitutes its own diagnostic entity. AML with mutations in splicing proteins (*SF3B1, SRSF2, U2AF1* and *ZRSR2*), certain epigenetic regulators (*EZH2, BCOR* and *ASXL1), RUNX1* or *STAG2* are associated with preceding MDS or MDS/MPN in at least 95% of cases. In the absence of AML-defining recurrent genetic abnormalities or a *TP53* mutation, mutations in these genes classify the disease as AML with myelodysplasia-related gene mutation. Cases with complex karyotype or myelodysplasia-related karyotypic changes but lacking *TP53* mutation or myelodysplasia-related gene mutations are classified as AML with myelodysplasia-related cytogenetic abnormality. Finally, cases not meeting criteria for any of the above groups are classified as AML not otherwise specified, regardless of their phenotypic features. This decision tree is shown graphically in Figure 32.5.

Although AML can develop through several biological trajectories (*de novo*, therapy-related, secondary to prior MDS or MPN or on the background of germline predisposition), genetic characteristics demonstrate the best correlation with disease prognosis and therefore provide the most relevance for disease classification. Current AML classification applies the biological context within which AML develops as a diagnostic qualifier to the AML-defining lesions. Therapy-related AML includes exposure to previous radiotherapy, cytotoxic therapy or immune-therapy for non-myeloid malignancy and constitutes around 10–15% of AML cases. Notably, germline mutations do occur in the therapy-related setting and taking a detailed family history as well as a full diagnostic work-up remains important. Prior MDS or MDS/MPN are added as

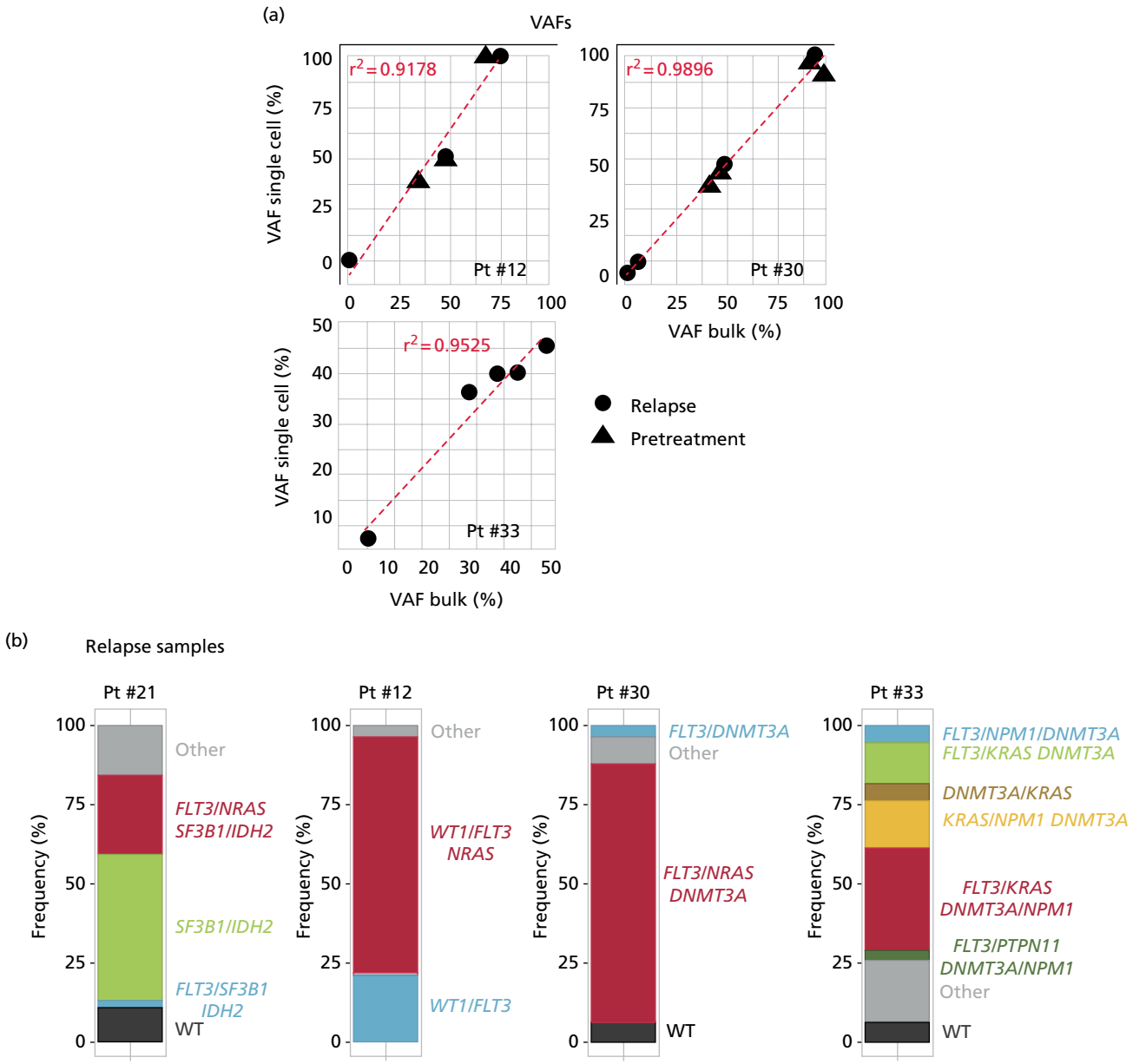

Figure 32.4 Examples of different patterns of genetic clonal evolution. *Patient #12* exhibits linear clonal evolution where at diagnosis only single AML clone exists bearing *FLT3* and *WT1* mutations. Early acquisition of *NRAS* mutation in this clone leads to treatment resistance and expansion of FLT3/WT1/NRAS clone. *Patient #30* exhibits branched evolution where selective pressure from gilteritinib leads to the suppression and disappearance of dominant *FLT3/DNMT3A* clone but permits the expansion of resistant *FLT3/DNMT3A/NRAS* subclone, which is present at low levels at the time of diagnosis. *Patient #21* exhibits both branched and linear polyclonal evolution. Acquisition of *NRAS* mutations bypasses the FLT3 inhibition effect of silencing MAPK-RAS signalling. *SF3B1/IDH2* clones expands under the selective advantage of FLT3 inhibition (Source: Taken from McMahon *et al. Cancer Dis.* 2019 https://cancerdiscovery.aacrjournals.org/content/9/8/1050.) (*Continued*)

qualifiers if they were diagnosed using standard diagnostics more than 3 months prior to the development of AML. Germline predisposition should be considered for AML patients of all ages, but especially if a person had 2 or more malignancies, AML is diagnosed at a young age, there is a family history within two generations of other haematological malignancy or solid malignancy at younger age (below 50) or in the presence of specific mutations.

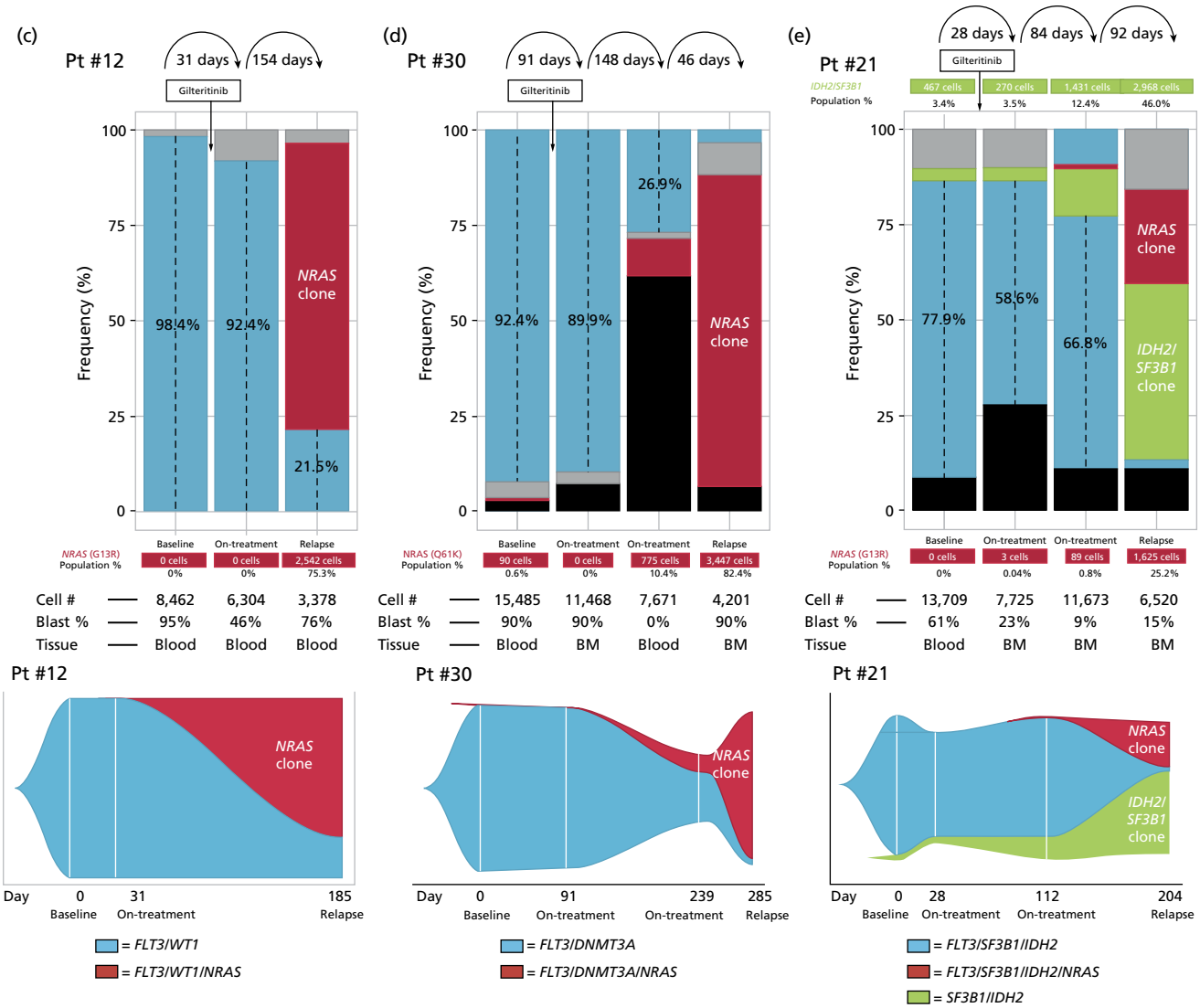

Figure 32.4 (*Continued*)

Diagnostic workup

An optimal diagnostic approach to AML is outlined in Table 32.3.

Recognition of familial myeloid neoplasms requires that physicians take a thorough patient and family history to assess for typical signs and symptoms, including data on malignancies, prior treatment and previous bleeding episodes.

If possible, patient should be consented for biobanking for wide indications and samples from diagnosis and various time points in treatment should be stored. Permanent infertility develops almost invariably with intensive AML treatments. Patients should be explicitly informed about this toxicity and should be offered cryopreservation of oocytes or sperm before treatment.

Flow cytometry of bone marrow aspirate or peripheral blood (if high circulating blast count) is essential for AML diagnosis and also establishes leukaemia-associated immunophenotype (LAIP), which can later be used for minimal residual disease (MRD) tracking. Baseline qPCR testing for NPM1 mutated, *CBFB::MYH11* or *RUNX1::RUNX1T1* AML is useful for later MRD disease tracking.

Prognostication

AML patients can have widely differing outcomes, that range from death within a few days of presentation to complete cure. The main cause of death for AML patients is treatment resistance: failure to achieve remission (refractoriness) or, more commonly, relapse. The genetics of individual AML cases can assign patients to discrete prognostic categories and is the

Table 32.2 ICC classification of AML and related neoplasms.

AML with recurrent genetic abnormalities (requiring ≥10% blasts in BM or PB)	**Myeloid sarcoma**
• APL with t(15;17)(q24.1;q21.2)/*PML::RARA**	*Acute leukaemia of ambiguous lineage*
• AML with t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1*	• Acute undifferentiated leukaemia
• AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11*	• MPAL with t(9;22)(q34.1;q11.2)/*BCR::ABL1*
• AML with t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A***	• MPAL with t(v;11q23.3)/*KMT2A*-rearranged
• AML with t(6;9)(p22.3;q34.1)/*DEK::NUP214*	• MPAL, B/myeloid, not otherwise specified
• AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM*(EVI1)****	• MPAL, T/myeloid, not otherwise specified
• AML with other rare recurring translocations*****	
• AML with mutated *NPM1*	
• AML with in-frame bZIP mutated *CEBPA*†	
• AML with t(9;22)(q34.1;q11.2)/*BCR::ABL1*‡	
Categories designated AML (if ‡20% blasts in BM or PB) or MDS/AML (if 10–19% blasts in BM or PB)	*Myeloid proliferations related to Down syndrome*
• AML with mutated *TP53*	• Transient abnormal myelopoiesis associated with Down syndrome
• AML with myelodysplasia-related gene mutations (*ASXL1*, *BCRO*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2. U2AF1*, *ZRSR2*)	• Myeloid leukaemia associated with Down syndrome
• AML with myelodysplasia-related cytogenetic abnormalities†††	*Blastic plasmacytoid dendritic cell neoplasm*
• AML not otherwise specified	

BM, bone marrow; MPAL, mixed phenotype acute leukaemia; PB, peripheral blood.
* *RARA* has also other rare translocations causing APL, which should be reported. For example *IRF2BP2::RARA*, *NPM1::RARA*, *ZBTB16::RARA*, cryptic inv(17), *STAT5B::RARA*, *STAT3::RARA*, *TBL1XR1::RARA*, *FIP1L1::RARA*, *BCOR::RARA*.
** KMT2 has over 70 translocation partners in AML and should be reported. The commoner ones are *AFF1::KMT2A*, *AFDN::KMT2A*, *MLLT10::KMT2A*, *TET1::KMT2A*, *KMT2A::ELL*, *KMT2A::MLLT1*.
*** There are other recurring translocation events in AML involving *MECOM* and should be reported accordingly, eg. AML with t(3;8)(q26.2;q24.2)/*MYC*, *MECOM*.
**** Other rare recurring translocations: AML with t(1;3)(p36.3;q21.3)/*PRDM16::RPN1*; AML (megakaryoblastic) with t(1;22)(p13.3;q13.1)/*RBM15::MRTFA*; AML with t(3;5)(q25.3;q35.1)/*NPM1::MLF1*; AML with t(5;11)(q35.2;p15.4)/*NUP98::NSD1*; AML with t(7;12)(q36.3;p13.2)/*ETV6::MNX1*; AML with t(8;16)(p11.2;p13.3)/*KAT6A::CREBBP*; AML with t(10;11)(p12.3;q14.2)/*PICALM::MLLT10*; AML with t(11;12)(p15.4;p13.3)/*NUP98::KMD5A*; AML with *NUP98* and other partners; AML with t(16;21)(p11.2;q22.2)/*FUS::ERG*; AML with t(16;21)(q24.3;q22.1)/*RUNX1::CBFA2T3*; AML with inv(16)(p13.3q24.3)/*CBFA2T3::GLIS2*.
† Monoallelic or biallelic.
‡ *BCR::ABL1* AML requires ≥20% of blasts to avoid overlap with CML in accelerated phase. History of previous CML precludes diagnosis of AML with *BCR::ABL1* and should be classified as CML in blast phase if PB or BM blast count is ≥20%.
††† Complex karyotype (≥3 unrelated chromosome abnormalities, excluding hyperdiploid karyotypes without structural abnormalities) or unrelated clonal abnormalities (del(5q)/t(5q)/add(5q); -7/del(7q); -8; del(12p)/t(12p)/(add)(12p); i(17q), -17/add(17p) or del(17p); del(20q); and/or idic(X)(q13).
(*Source:* Adapted from Arber *et al. Blood* 2022 https://ashpublications.org/blood/article/140/11/1200/485730/International-Consensus-Classification-of-Myeloid.)

most powerful prognostic factor, explaining two-thirds of the variability in overall survival (OS). Other important disease characteristics relate to whether the disease is primary or secondary and to the haemoglobin, platelet and white blood cell count and peripheral blood blast percentage. Patient-specific characteristics not related to the disease are also important prognostic factors, with the most important being ECOG/WHO performance status, creatinine and albumin. Age in itself reflects a composite of multiple prognostic cofactors, such as performance status, comorbidities, a higher chance of therapy-related AML or AML developing from myelodysplasia/MPN and an increased frequency of potentially high-risk mutations (e.g. *RUNX1, ASXL1, BCOR, STAG2, USAF1, ZRSF2, SRSF2, SF3B1* and *EZH2*). Removal of age from the AML prognostication models did not significantly alter the accuracy of prediction. Therefore, patient's age should be considered only secondary to other factors when decisions about treatment intensity are made.

In 2022, the European Leukaemia Network (ELN 2022) updated a risk-stratification framework, based on genetics, which

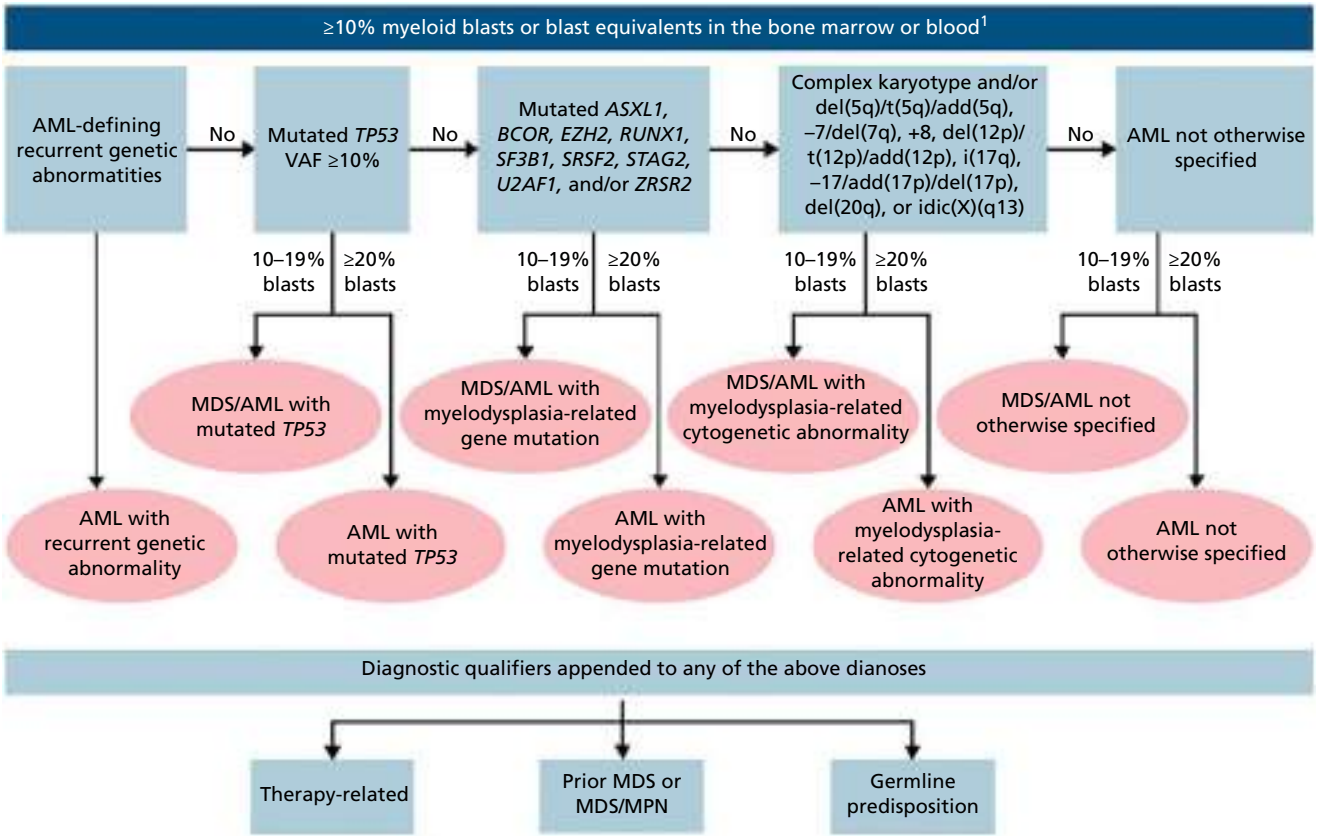

Figure 32.5 Hierarchical approach to the classification of AML from the International Consensus Classification 2022. Germline predisposition qualifier should include the name of the mutated germline gene or syndrome. Examples of how diagnostic qualifiers are addended: 1. *AML with in-frame mutation in the bZIP domain of the CEBPA gene, germline CEBPA mutation* 2. *AML with mutated TP53, therapy-related; AML with myelodysplasia-related gene, prior myelodysplastic syndrome* (Source: Döhner *et al. Blood* 2022).

Table 32.3 Diagnostic workup of AML.

History	• Comorbidities including prior malignancy (personal history of ≥2 cancers, one of which haematological, is indication for germline testing)
	• Bleeding history to identify germ line predisposition to platelet and myeloid disorders
	• Family history to identify germline myeloid neoplasms:
	  ○ Another relative within two generations with another haematopoietic malignancy, or
	  ○ Another relative within two generations with a solid tumour diagnosed at age 50 or younger, or
	  ○ Another relative within two generations with other haematopoietic abnormalities
	• ECOG performance status or WHO score
	• Pre-existing haematological disorder
	• History of chemotherapy, immunotherapy or radiotherapy treatment, smoking, benzene exposure

Examination	Full clinical examination with particular consideration for presence and source of infection, skin (*leukaemia cutis*, thrombocytopenia and Sweet syndrome), CNS involvement, testicular involvement, syndromic feature that would suggest germline predisposition
Blood tests	• Full blood count • Blood film (blast count from at least 200 leucocytes) • Biochemistry (creatinine, urea, sodium, potassium, calcium, phosphate, magnesium, alanine transferase, alkaline phosphatase, bilirubin, albumin, glucose, lactate dehydrogenase, uric acid and total cholesterol) • Clotting (PT, APTT ± INR) • Hepatitis A, B, C, HIV-1, EBV, CMV, VZV, HSV screen • *If potential allogeneic stem cell transplant candidate*: CMV IgG before first transfusion, HLA genotyping and donor search

(*Continued*)

Table 32.3 (*Continued*)

Other tests	• ECG • Chest Xray • Echocardiogram (especially if anthracycline therapy considered) • Pregnancy test (in females of childbearing age) • Biobanking • Lumbar puncture if clinical and/or radiological suspicion of CNS disease	
Bone marrow aspirate ± trephine biopsy,***		
Morphology	Blast count from at least 500 cells from bone marrow smear	
Immunophenotyping	Panel should be comprehensive enough to distinguish AML, B-ALL, T-ALL, early precursor T-ALL and acute leukaemias of ambiguous lineage.	
	Diagnosis of AML. Cell-surface and cytoplasmic markers	
	Precursors	CD34, CD117 and HLA-DR
	Myeloid markers	CD33, CD13 and cytoplasmic MPO
	Myeloid maturation markers	CD11b, CD15, CD65 and CD64,
	Monocytic markers	CD14, CD36, CD64, CD11c, CD4 and CD38
	Megakaryocytic markers	CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa) and CD36
	Erythroid markers	CD235a (glycophorin A), CD71 and CD36
	Diagnosis of MPAL	
	Myeloid lineage	MPO (flow cytometry, immunohistochemistry, or cytochemistry) or monocytic differentiation (at least 2 of the following: nonspecific esterase cytochemistry, CD11c, CD14, CD64 and lysozyme) or at least 2 myeloid markers
	T-lineage	Strong cytoplasmic CD3 (with antibodies to CD3 ε chain) or surface CD3
	B-lineage	Strong CD19 with at least 1 of the following strongly expressed: cytoplasmic CD79a, cCD22 or CD10 or weak CD19 with at least 2 of the following strongly expressed: CD79a, cCD22 or CD10
	Core MRD markers	
	CD34, CD117, CD45, CD33, CD13, CD56, CD7 and HLA-DR	
	If monocytic, additionally: CD64, CD11b and CD4	
Genetic tests	Cytogenetics	AML karyotype (at least 20 metaphases)
		FISH/PCR for *CBFB::MYH11* [inv(16)(p13.1q22)], *RUNX1::RUNX1T1* [(8;21)(q22;q22.1)], *BCR::ABL1* t(9;22)(q34.1;q11.2) and other available fusion genes. Specific FISH panels are available for paediatric AMLs.
		If suspicion of APML: FISH/PCR *PML::RARA* [t(15;17)(q24.1;q21.2)]
	Mutation screening***	Essential to identify targetable lesions (*FLT3::ITD*, *FLT3::TKD* hotspot, *IDH1* and *IDH2*) and necessary for diagnostic accuracy: *NPM1*, *CEBPA* (specifying whether in frame of bZIP or other mutation), *DDX41*, *TP53*, *ASXL1*, *BCOR*, *EZH2*, *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2*
		Desirable to identify potential MRD targets or CH origin of the disease:
		ANKRD26, *BCORL1*, *BRAF*, *CBL*, *CSF3R*, *DNMT3A*, *ETV6*, *GATA2*, *JAK2*, *KIT*, *KRAS*, *NRAS*, *NF1*, *PHF6*, *PPM1D*, *PTPN11*, *RAD21*, *SETBP1*, *TET2* and *WT1*
	Whole genome sequencing	Tumour (bone marrow aspirate or peripheral blood with high blast count) AND
		germline (skin biopsy preferred; saliva or buccal swab acceptable if blasts cleared from PB)

CMV, cytomegalovirus; EBV, Epstein Barr virus; HSV, herpes simplex virus; MPAL, mixed phenotype acute leukaemia; MPO, myeloperoxidase; NGS, next generation sequencing; VAF, variant allele frequency; VZV, varicella zoster virus.

* Trephine biopsy compulsory if no aspirate obtained ('dry tap') – perform trephine roll or touch preparations on the slide. Tissue biopsy of extramedullary disease if no apparent bone marrow or peripheral blood involvement.

** Peripheral blood is accetable alernative if high blast count ($>2 \times 10^6$/mL) and aspirate not feasible.

*** NGS myeloid panels are becoming more mainstream and part of upfront investigations.

(*Source:* Adapted from Döhner *et al. Blood* 2022.)

Table 32.4 2022 ELN AML risk stratification by genetics.

Risk category	Genetic abnormality
Favourable	t(8;21)(q22;q22.1)/*RUNX1::RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB::MYH11* Mutated *NPM1* without *FLT3::ITD* bZIP in-frame mutated *CEBPA*
Intermediate	Mutated *NPM1* with *FLT3::ITD* Wild-type *NPM1* with *FLT3::ITD* (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3)/*MLLT3::KMT2A* Cytogenetic and/or molecular abnormalities not classified as favourable or adverse
Adverse	t(6;9)(p23.3;q34.1)/*DEK::NUP214* t(v;11q23.3)/*KMT2A*-rearranged excluding *KMT2A* partial tandem duplication t(9;22)(q34.1;q11.2)/*BCR::ABL1* t(8;16)(p11.2;p13.3)/*KAT6A::CREBBP* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)* t(3q26.2;v)/*MECOM*(*EVI1*)-rearranged −5 or del(5q); −7; −17/abn(17p) Complex karyotype, monosomal karyotype Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1* and/or *ZRSR2* in the absence of favourable risk mutations Mutated *TP53*

(*Source:* Taken from Döhner *et al. Blood* 2022.)

is currently the most widely adopted system for pre-treatment AML prognostication. It classifies patients into favourable-, intermediate- and adverse- risk categories based on the underlying genetic abnormalities (Table 32.4). Notably, early classification of *NPM1*-mutated, *RUNX1::RUNX1T1*, *CBFB::MYH11 or KMT2A-rearranged AML* can be modified by MRD status during treatment. *NPM1*-mutated AML with adverse risk cytogenetic abnormalities reclassifies AML into adverse risk. Adverse risk cytogenetic abnormalities exclude multiple trisomies (or polysomies). Patients with *CBFB::MYH11* or *CEBPA* mutation(s) have very favourable prognosis with 5-year OS of around 70%. In contrast, *TP53* mutations are associated with dismal outcomes and in combination with complex karyotype constitutes the subtype of AML with the worst prognosis.

The impact of genetic factors on AML prognosis is combinatorial and may be modified by the co-occurrence of other genetic abnormalities (Figure 32.6). However, next generation sequencing (NGS) studies suggest that the ELN 2022 genetic prognostication framework, which largely considers only a single genetic alteration (with the exception of *FLT3* and *NPM1* co-mutation), is likely too simplistic, given that an average case of newly diagnosed AML has 3–5 acquired mutations. Current and future work are trying to develop better classification and prognostication systems that take into account not only the genetic combinatorial diversity of AML, but also evidence-based individually tailored prognostication and treatment decisions. These require large databases (*'knowledge banks'*) of thousands of patients that link disease genomics and clinical data. Utilisation of a knowledge bank approach has already been demonstrated to provide superior predictions of likelihood of remission, relapse and mortality at individual level, as compared with standard prognostication methods. International effort is underway to compile such systematised databases.

Although pre-treatment genetics provide powerful prognostic information, these are static and do not take into account subsequent variable such as treatment-response. It is likely that combination with dynamic measures, such as minimal residual disease (also known as measurable residual disease, both MRD) will further refine clinical decision-making tools to aid personalised and optimal therapy in AML.

Treatment

Treatment aspirations

The first decision in managing a patient with AML is to set individualised treatment goals. Given the wide distribution of patients across the spectrum of age and performance-status, these goals range from simple supportive care all the way to full curative intent. In addition, these goals might alter during treatment and need to be continuously reassessed, taking into account the patient's wishes at every stage. There is little doubt, based on the evidence from several multicentre trials, that in younger patients (<65 years) with no or little comorbidities an intensive therapy approach aiming to cure provides longer-term benefit. However, as only a selected minority of older patients (>65 years) have been historically entered into trials of intensive therapy, the trial-derived information regarding intensive therapy is less likely applicable in this age group. Moreover, given disease biology is often less favourable in elderly patients and the majority of older patients are considered unfit for intensive therapy because of comorbidities, a therapeutic approach that carries a reduced risk of toxicity, preserves quality of life while still providing a degree of disease control is preferable. Although historically this has often meant a palliative treatment approach, based on supportive care with limited low-intensity therapeutic options, the recent development of newer more targeted therapies has started to change life expectancy across all AML patients. Randomised phase 3 trial evidence of prolonged survival has recently been demonstrated in elderly AML patients, although chances of long-term cure still remain low.

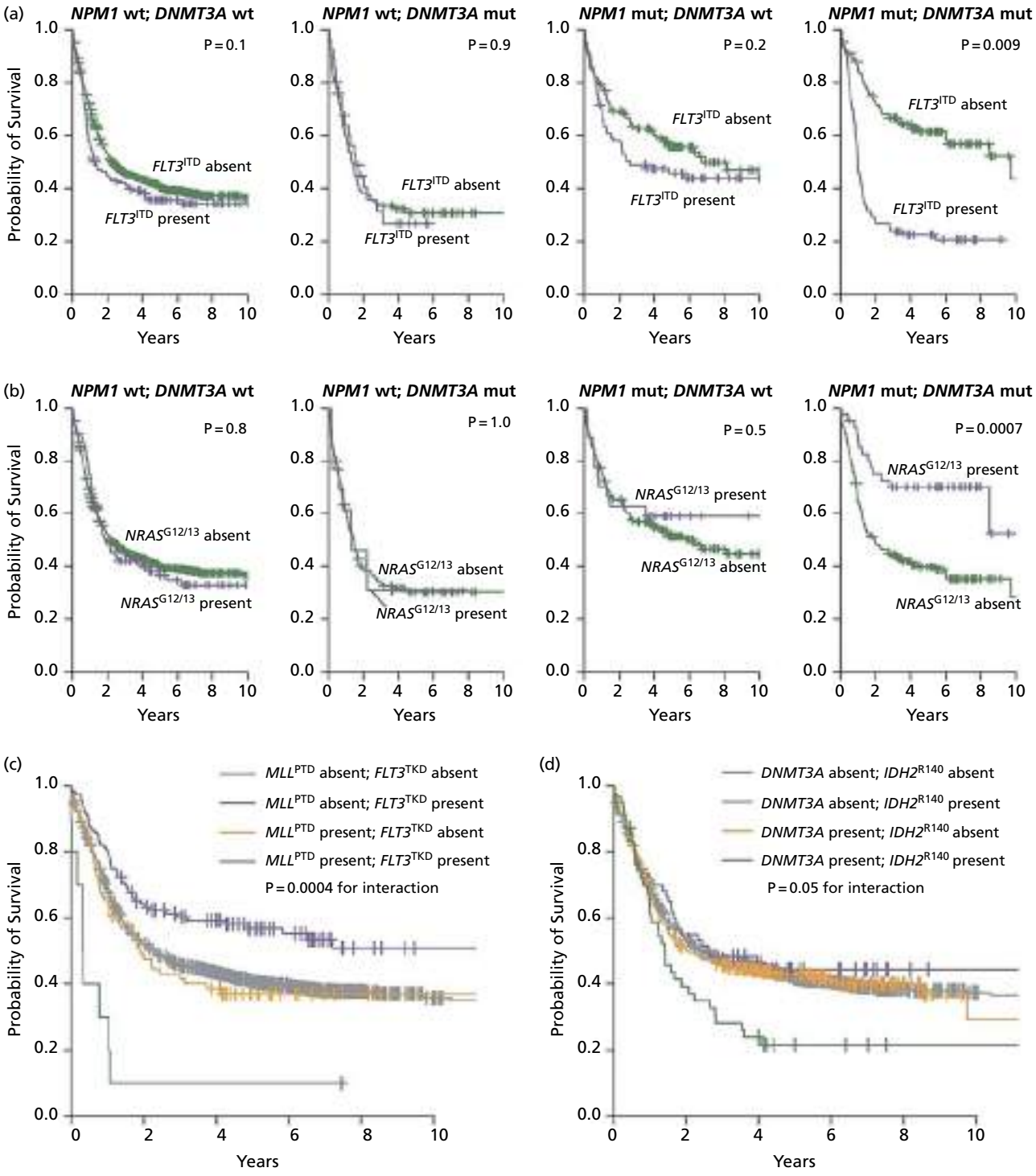

Figure 32.6 Examples of the influence of gene-gene interaction for AML prognosis. (a) *NPM1* and *FLT::ITD* mutated AML have significantly worse prognosis with co-mutated *DNMT3A* than with wild-type *DNMT3A*. (b) *NPM1* and *NRAS* mutated AML have significantly better prognosis with co-mutated *DNMT3A* than with wild-type *DNMT3A*. (c) Partial tandem duplication (PTD) of the MLL gene and *FLT3* tyrosine kinase co-mutation result in very poor prognosis compared to these aberrations occuring in isolation. (d) *DNMT3A* and $IDH2^{R140}$ co-mutation results in poorer prognosis compared to these aberrations occuring in isolation (Source: Taken from Papaemmanuil *et al. NEJM* 2016 https://www.nejm.org/doi/full/10.1056/nejmoa1516192.)

Treatment strategy

The first aim in AML therapy is to reduce disease bulk, aiming to classically achieve a complete (morphological) remission (CR) and thus allow the recovery of normal bone marrow function. Achieving CR approximately reduces tumour burden by 2 logs (from ~10^{12} to 10^{10} leukaemic cells) and is defined as the achievement of <5% blasts in a cellular marrow durable for at least 28 days with a peripheral neutrophil count of $\geq 1 \times 10^9$/L and platelet count above 100×10^9/L and absence of extramedullary disease. When CR is not associated with full peripheral count recovery, a CRi (CR with incomplete count recovery) is attained. CRi may reflect a pre-existent dysplastic state or the effect of over-treatment and has been shown to be associated with reduced OS. However, morphological assessment of remission has low sensitivity and is now routinely complemented by the use of more sensitive techniques to assess residual disease, such as flow cytometry and molecular monitoring of AML-associated mutations/chromosomal aberrations, which are respectively capable of detecting residual malignant cells at a level of 1 in 10^4 – 1 in 10^{6-7}. Although these techniques are not always applicable, evidence is now compelling that achievement of deeper response, i.e. MRD negative (MRD−), is associated with better long-term outcomes, suggesting achievement of a MRD− CR should be the optimal aim of induction therapy.

Following induction therapy, clinical experience has demonstrated that further consolidation therapy is required to reduce relapse risk. The choice of consolidation therapy to be used in an individual patient is guided by a variety of clinical and biological features and will likely vary between patients. This usually consists of one or more of several options such as further blocks of intensive therapy, stem cell transplantation or in some instances continuous maintenance therapy.

Induction

The choice of induction therapy depends on several factors, i.e. disease biology, patient's age, comorbidities and preferences. The recent approval of less toxic more targeted therapies has also expanded the options available, particularly for less fit patients. The option of entering patients into clinical trials should always be considered. These trials often also provide access to novel therapies in combination with standard approved drugs to improve CR ± MRD rates and trial entry has been shown to consistently result in better patient long-term OS.

Standard intensive chemotherapy (IC)

The standard backbone of induction therapy for young, fit AML patients has not really changed across the last 40 years and consists of a combination of daunorubicin and cytarabine (DA). Historically daunorubicin has been given for 3 days at a dose of 45–60 mg/m^2, with Cytarabine (Ara-C) given for 7–10 days as a continuous infusion or by bolus doses of 100–200 mg/m^2 daily ('7 + 3'). Many clinical trials have been conducted to test variations on this standard of care. Other anthracycline alternatives to daunorubicin (doxorubicin, mitoxantrone, idarubicin and aclarubicin) or different doses are yet to demonstrate consistent superiority. Particularly, recent studies have explored the role of intensification of the daunorubicin dose to 90 mg/m^2, but no obvious superiority has been shown. Similarly, higher doses of Cytarabine have also failed to demonstrate a consistent benefit. Regarding optimal delivery of these two drugs, CPX-351 (Vyxeos) has shown promising results in AML subgroups as will be discussed later. An alternative induction regimen is the FLAG-Ida combination (fludarabine/Ara-C/G-CSF and idarubicin). This increased intensity regimen results in reduced relapse rates, but generally at the expense of increased and more prolonged myelosuppression.

Using the treatment approaches outlined above, 75–85% of patients under 60 years and 50–60% of older patients will enter morphological CR. Of those that do, about 70% will have required one course to achieve CR. If an incomplete response is obtained, then a second course of the same combination is indicated. If patients fail to achieve a substantial reduction in marrow blasts (to <15% blasts) in the first course or fail to enter CR with a second course, they should be considered refractory to the drugs used up to that point and alternative treatment options, ideally a clinical trial of agents working through different mechanisms, should be considered. Nevertheless, these patients have an overall higher risk of rapid relapse and would immediately be candidates for allogeneic stem cell transplant (SCT) at consolidation. Assessment of MRD status post-induction has recently emerged as an important adjunct to define depth of response and remission status with important implications for the choice of consolidation therapy.

Several factors influence the prospects of achieving remission – with age, performance status and poor-risk disease being perhaps the strongest. However, as performance status and poor-risk disease are significantly linked with age, it is difficult to fully separate out these variables. Conceptual criteria proposed by an Italian panel of experts (Ferrara fitness criteria) have been shown to accurately predict fitness for intensive chemotherapy. Treatment-related mortality (TRM) rates rise from <5% to around 15% in Ferrara fit and unfit patients respectively, with a subsequent effect on long-term OS of patients. Improvement in supportive care and the introduction of less intensive but still effective therapy might reduce the incidence of TRM which remains an inevitable but highly unsatisfactory outcome of induction therapy for a proportion of patients.

The increased understanding of AML molecular features and biology has led to the development of novel therapies such as small inhibitors targeting activating *FLT3*-mutations and neomorphic *IDH*-mutations (see later sections), therapies targeting

the antiapoptotic machinery of AML cells (BCL2 inhibitors) or antibody-drug conjugates targeting antigen expressed specifically on AML blasts such as CD33. Some of these have now been studied as an adjunct to standard daunorubicin/cytarabine IC schedules or as backbone for alternative induction therapy, mostly with positive results and have become standard of therapy in specific subgroup of patients.

Midostaurin and FLT3 inhibition

Midostaurin, an oral multitargeted tyrosine kinase inhibitor active in patients with activating *FLT3*-mutations (both TKD and ITD), has been studied as an adjunct to standard 7+3 induction in adults with *FLT3* mutations aged <60. In the multicentre RATIFY Trial, Midostaurin was added at a dose of 50 mg twice daily from day 8 to 21 during induction and 'consolidation' and then alone as 'maintenance' at the same dose for 1 year. Although CR rates were similar in the midostaurin and placebo groups, survival was longer with midostaurin, largely reflecting a lower relapse rate. The effects were seen regardless of ITD allelic ratio or type of *FLT3* mutation (ITD or a TKD). There was no difference in proportions of patients progressing to stem cell transplant between the two arms and Midostaurin superiority was also significant after censoring at time of transplant. Survival was superior with Midostaurin if SCT was performed in first complete remission (CR1) but not later suggesting that the beneficial effect of Midostaurin is lost following frank relapse. Overall, these data suggest that addition of Midostaurin is able to reduce relapse risk and improve survival in *FLT3*-mutant AML patient most likely through achieving deeper responses regardless of subsequent consolidation. Based on these data, the addition of Midostaurin to standard induction is now considered standard therapy for adults aged <60 with newly diagnosed AML and *FLT3* mutations and subsequent phase 2 trials suggest that similar benefit is seen also in patients >60 years old who are able to tolerate standard induction therapy. Recently more potent and specific FLT3 TKI such as Gilteritinib, Crenolanib and Quizartinib have entered clinical use with evidence of significant efficacy in the relapsed/refractory setting. These are now currently being combined with chemotherapy and compared to chemotherapy and Midostaurin, with results awaited.

Gemtuzumab ozogamicin

CD33 is a cell surface antigen commonly expressed on AML cells, and normal myeloid progenitors, including stem cells. Gemtuzumab ozogamicin (GO) is an antibody-drug conjugate (ADC) to CD33 conjugated with the toxin calicheamicin. GO (Mylotarg®; Pfizer) alone has shown high efficacy against acute promyelocytic leukaemia (APML) presumably because of the heavy expression of CD33 in APML. However, as a single agent, it is not particularly effective against other subtypes of AML. There has therefore been much interest in adding GO to induction treatment. Similar to Midostaurin, GO does not appear to improve CR rates, but reduces the risk of relapse and thereby improves survival in specific patient subgroups. A recent patient-based meta-analysis of the five randomised trials in adults where GO was added to induction confirms the overall reduction in relapse and the survival benefit specifically in non-adverse-risk patients. Interestingly, there does not appear to be a clear correlation between CD33 expression levels in AML and sensitivity to GO. Therefore, GO therapy is not assigned on the basis of CD33 expression levels. Based on these data currently GO is licensed for use in CD33 positive AML with favourable- or intermediate-risk profile and has become standard of therapy for this patient group. However, despite its efficacy in APML it remains unlicensed in this setting.

CPX-351 (Vyxeos)

CPX-351 (Vyxeos®; Jazz Pharmaceuticals, Inc.) is a liposomal encapsulation of cytarabine and daunorubicin at a fixed 5:1 synergistic molar ratio demonstrated to be optimal for anti-leukaemic activity. A recent Phase 3 trial in patients aged 60–75 and fit for intensive therapy, with either therapy-related, secondary AML or AML with MDS-like cytogenetic abnormalities demonstrated higher CR rates with CPX-351 compared to standard induction, which translated into improved long-term survival rates (5-year survival 18% in CPX-351 vs 8% in 7 + 3 group). Interestingly, Vyxeos was also better tolerated, with reduced early mortality rates. A higher proportion of patients proceeded to SCT after CPX-351 therapy, with these patients showing the largest benefit. Benefits were seen in some patients from high-risk groups such as those harbouring *RUNX1* and *ASXL1* mutations, however, other covariates such as poorer performance status, previous hypomethylating agents and adverse cytogenetics, particularly *TP53* mutations were associated with poorer rates of CR and survival with no obvious benefit from CPX-351. Based on the overall evidence from this phase 3 trial, CPX-351 is now approved for patients with therapy-related AML (t-AML) or AML with MDS-related changes regardless of age.

Induction therapy in the unfit patient – Venetoclax and hypomethylating agents (HMA)/LDAC

Previously, low-dose cytarabine (LDAC) at 20 mg/m^2 for 10 days every 28 days and HMA (Azacitidine and Decitabine given for 5–10 days every 4 weeks) have been the main therapies used in

patients unfit for intensive chemotherapy. HMA have shown superior efficacy compared to LDAC in terms of CR rates and durability of response, with Azacitidine providing a survival advantage compared to conventional care regimens (standard chemotherapy, LDAC or best supportive care) in elderly, >65 years old, AML patients in the AML001 trial. The recent development of Venetoclax, an oral inhibitor of the anti-apoptotic protein BCL-2, has further changed the treatment algorithms in this patient population. BCL-2, the founding member of the BCL-2 family of anti-apoptotic proteins, appears to be highly expressed in many AML subtypes and promotes AML cells survival and resistance to therapy. Although as a single agent Venetoclax appears to have limited efficacy, its combination with either HMA or LDAC is synergistic in eliminating AML cells. Two large phase 3 trials in AML across all age groups considered unfit for standard induction therapy, the VIALE-A and VIALE-C trial, have recently reported the efficacy of respectively, Venetoclax and Azacitdine and Venetoclax and LDAC, compared to single agent HMA or LDAC therapy. In both trials, the Venetoclax combinations provided higher CR rates and deeper responses which resulted in significant improvement in OS, most notable in the Venetoclax and Azacitidine arm. Interestingly Venetoclax combinations appeared to be particularly effective in specific subtypes of AML, i.e. those carrying *NPM1c* and *IDH* mutations, where MRD-negative remission have often been observed. Additionally, a plateau in survival curves could be observed at around 30–40% in the Venetoclax and Azacitidine (Ven/Aza) combination arm, although longer follow-up will be needed to draw definite conclusions. Similar to that is observed in patients treated with IC (see below), achievement of flow MRD negativity at any time-point following Ven/Aza is associated with improved OS of 84.6% at 18 months. In addition, this combination experimentally targets leukaemia stem cells (LSC), possibly through inhibition of their specific dependency on oxidative phosphorylative metabolism. Resistance to Venetoclax has been described in patients with mutations in signalling kinases/pathways such as *FLT3* and *RAS*, and durable responses are low in patients with *TP53*-mutations, despite their initial sensitivity, where they have a CR rate of over 50%. Although Ven/Aza is generally associated with lower TRM than standard intensive chemotherapy, Ven/Aza combinations are not without toxicity, in particular prolonged cytopenias. As such, these combinations are better defined as 'intermediate' rather than 'low' intensity and might not always be tolerable for the frailest patients. Moreover, they are currently offered as continuous therapy with attendant financial and compliance implications, although in reality, only a minority of patients are able to continue on therapy beyond 12 months. An MRD-directed treatment de-escalation has been suggested as possible in specific cases carrying sensitive genotypes following at least 12 cycles of therapy but will require further studies to be fully supported in routine clinical practice. Whether such combination could provide similar or even improved disease control in the more sensitive subtypes of AML compared to standard IC is currently being addressed in clinical trials including in the UK in *NPM1c* mutant AML through the VICTOR study. Finally, the addition of other novel therapies such as FLT3 inhibitors to the Ven/Aza backbone is currently being trialled in early-phase trials with very positive response rates although at the expense of increased toxicity. Moreover, a recent phase 3 trial of the FLT3 inhibitor Gilteritinib in combination with Azacitidine versus Azacitidine on its own failed to show improved survival rates, despite increased responses in newly diagnosed FLT3 mutant AML patients unfit for IC. As a result, Venetoclax/HMA remains the standard of care for all AML patients unfit for IC currently.

Consolidation therapy

Following the achievement of remission, the priority in AML patients is to prevent subsequent relapse. While optimisation of induction treatment will also affect depth of remission and subsequent risk of relapse, historical experience has shown us that patients require consolidation therapy for full curative intent. The options for consolidation therapy are either further chemotherapy at a similar intensity level to induction therapy and/or chemo/chemoradiotherapy followed by allogeneic SCT or rarely, in selected cases, autologous SCT. More recently, maintenance therapy has also started to be used in specific group of patients. The choice of consolidation therapy is arguably the most important one in the therapeutic journey of AML patients. It has to balance the risk of relapse against the TRM and cumulative morbidity associated with further chemotherapy and transplantation. Historically, prognostic features at diagnosis have been used to estimate relapse risk in individual patients; however, more recently response to therapy via MRD monitoring has been shown to be the best predictor of outcome in many instances, and currently a combination of both prognostic markers at diagnosis and MRD assessment at informative time points is used to guide decisions alongside patient-specific factors. Although this represents a first significant step towards precision medicine in AML, considerable inter-patient heterogeneity in driver mutations and depth of response still renders evidence-based personalisation of care challenging. In addition, current ELN criteria are mostly relevant to the selection of postremission therapies for younger, fit and transplant-eligible patients with *de novo* AML treated with conventional cytarabine/anthracycline-based induction. They have less relevance for elderly patients as they do not take relevant clinical features into account, nor do they capture clinical/biological predictor of response to novel therapies such as venetoclax. As a result, they fail to properly categorise many adverse-risk patients. Several efforts recently have been made to leverage 'big data' from thousands of AML patients to study how large

knowledge banks of matched genomic–clinical data can help clinical decision-making, with large international efforts such as the HARMONY consortium aiming to capture the entire heterogeneity of AML through studies involving >10,000 patients. Such studies would be very welcome clinical decision support tools to inform individualised patient treatment choices.

MRD risk-adapted therapy

A key feature of risk stratification that has recently been clearly established is the assessment of individual patient response using highly sensitive techniques to measure MRD. MRD assessment is now becoming standard-of-care to guide risk-adapted consolidation therapy with the dual aims of a reduction in toxicity and an optimisation of response. Multiple studies have shown significant correlation between MRD quantified by molecular techniques (RT-qPCR and NGS) or multiparameter flow cytometry (MFC) and outcome. MRD assessment provides further refinement to risk prediction and can be applied to several aspects of the post-remission decision-making process: (i) assessment of the quality of response to improve risk stratification and enhance postconsolidation choice (e.g. SCT versus no-SCT); (ii) post-treatment serial monitoring to anticipate overt relapse and plan pre-emptive therapy; (iii) pre-SCT assessment and post-transplant surveillance monitoring to assess the risk of relapse, inform immunosuppression and the utilisation of donor lymphocyte infusions; and (iv) identification of markers to track treatment response in the context of targeted-therapy approaches.

MRD Modality – Molecular and flow cytometry are the most frequently used modalities to monitor MRD; however, NGS is also being increasingly adopted. Molecular testing by RT-qPCR provides a robust, highly sensitive (generally 1 AML cell in 10^{6-7} normal cells in BM and one log less in peripheral blood) and standardised assay. It can, however, only be used in around 50% of cases that have an informative leukaemia-specific genetic marker (i.e. fusion gene, *NPM1* mutation, etc.) amenable to MRD detection. Flow cytometry MRD is based on the identification at diagnosis of leukaemia-associated aberrant immunophenotypes (LAIPs). These can be described in the majority, around 90%, of cases at diagnosis. These LAIPs are characterised by expression of antigens not normally seen in combination on normal haemopoietic cells; either aberrant antigen expression or co-expression of antigens normally associated with different stages of maturation or absence of specific antigens expression. The greatest advantage of flow-cytometry-based MRD monitoring is its applicability to the vast majority of AML cases, although sensitivity tends to fall below molecular monitoring at 1 in 10^4 or 1 in 10^5 cells. Standardisation also remains an issue with flow MRD, although this is likely to improve over the coming years. NGS can, theoretically, be applied to all leukaemia-specific genetic aberrations. However, not all genes are suitable for MRD detection, either because they may be lost at relapse (eg, *FLT3*) or because they are associated with age-related clonal haematopoiesis and may represent pre-leukaemic clones rather than true MRD (*DNMT3A, TET2, ASXL1*). Standardisation of NGS MRD assessment is also required to support its widespread clinical use and currently, it remains mostly used within clinical studies.

MRD assessment can be performed at different time points, i.e. after induction and achievement of CR or after completion of consolidation therapy and subsequently to monitor for molecular relapse. The best timepoint depends on the risk profile of specific AML patients and often sequential MRD assessment at different times, focusing on an increase or decrease rather than a single result, is necessary before actioning therapeutic changes. Despite these caveats, MRD positivity after induction has been clearly demonstrated to carry a high positive predictive value for subsequent morphologic relapse, regardless of pre-treatment risk group allocation. For intermediate-risk AML, where individual patient risk profile can be quite variable, MRD has been clearly shown to separate patients into two groups with very different OS long-term. MRD positivity using RT-qPCR for *NPM1c* mutations in patients with intermediate-risk AML in CR after two courses of induction treated within UK NCRI AML trials have been shown to be highly predictive for subsequent relapse risk, with a 86% cumulative incidence of relapse vs 34% for MRD negative patients (Figure 32.7). Multivariate analysis indicated persistent *NPM1c* positivity in blood was the only predictor of relapse regardless of *FLT3* mutation status.

Similarly, for patients without *NPM1c* mutations, MFC MRD after course 2 was able to dichotomise a low- and high-risk group, although the separation was less marked (cumulative incidence of relapse was 89% for MRD >0.1%, 58% for MRD positive <0.1 and 50% for MRD negative). Based on these data, there is now wide support in the clinical community to change therapeutic approaches in patients with intermediate-risk AML at diagnosis and MRD positivity after induction and consider allogeneic transplantation in first remission where possible. In favourable-risk AML such as core-binding factor mutants, MRD positivity after induction is also associated with increased relapse risk, often up to 50%, although given their continued chemosensitivity and the risk-benefit ratio, this patient group is usually not considered for SCT in CR1. MRD positivity before allogeneic transplant has also been associated with worse outcome; however, there is some evidence that achieving rapid donor chimerism, possibly through modulation of transplant conditioning and/or immunosuppression, might abrogate the adverse impact of pre-transplant MRD. Finally, although it is conceivable that MRD negative response correlates with deeper

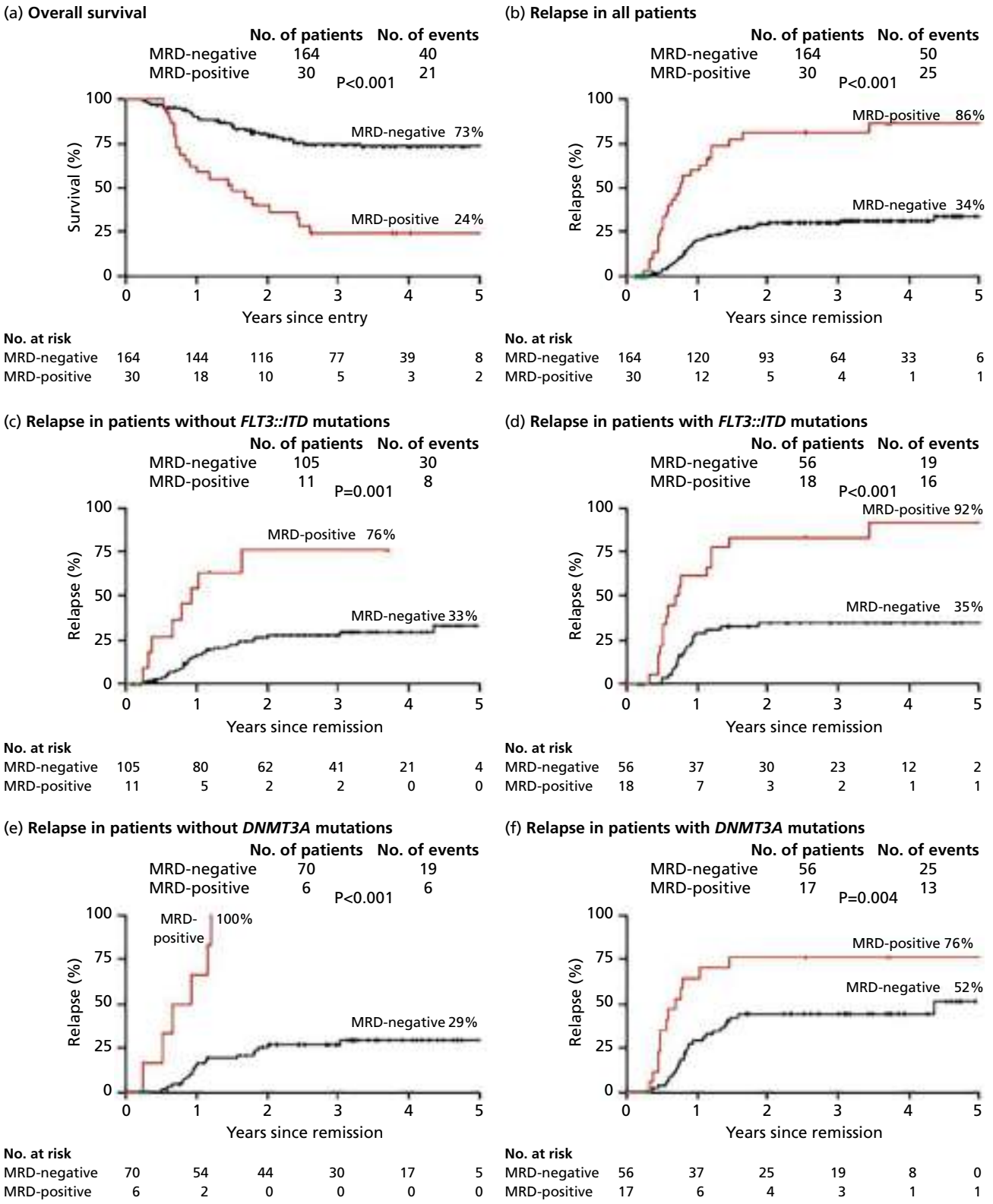

Figure 32.7 Utility of MRD analysis in NPM1-mutated AML patients. Panels (a) and (b) demonstrate the predictive role of MRD-positivity in defining patient survival (a) and risk of relapse (b). How these risks are further modified by co-mutation with *FLT3::ITD* and *DNMT3A* are shown in panels (c) and (d) and (e) and (f) respectively (Source: Reproduced from Dillon *et al.* Blood 2020/American Society of Haematology.)

remissions and better patient outcomes regardless of therapy used, it is noticeable that the prognostic value of MRD monitoring has been mostly studied in patients treated with intensive standard induction chemotherapy and therefore our ability to extrapolate these findings onto patients treated with different approaches, such as novel intermediate intensity therapy will require further validation.

Consolidation treatment – chemotherapy

Optimal consolidation therapy should be an individualised decision based on patient-specific risk profiles, including response to therapy at the morphological and MRD level. The options available are further chemotherapy at the same level of intensity of induction or chemo/radiotherapy followed by allogeneic SCT, or in a small number of cases, autologous SCT. More recently, maintenance therapy has also been shown to be beneficial in specific settings following both standard consolidation chemotherapy or transplant.

Chemotherapy will usually involve a further one or two courses containing high-dose Ara-C (generally 3 g/m^2 twice daily on alternate days over 5 days). Higher doses of cytarabine appear particularly beneficial for patients with more sensitive disease, such as those with favourable cytogenetics. UK NCRI trials have also shown that reducing the Ara-C dose to 1.5 g/m^2 might be as effective, with a more favourable toxicity profile, and currently, most centres will use this dose routinely in patients >60. More recently, the NCRI group has also assessed whether reducing consolidation to 1 single course following two induction courses is possible without prejudicing outcome. Although there was no significant survival advantage by giving two consolidation courses of high-dose Ara-C, 5-year relapse rates were significantly lower following two courses of consolidation (58 versus 50%). Moreover, subset analysis showed that patient with more favourable and chemosensitive disease might especially derive survival benefit from two consolidation courses. Based on this and given the use of MRD to adapt therapy in less chemosensitive patients, it seems appropriate to use two courses of consolidation as standard consolidation in patients showing deep response to standard chemotherapy, unless this course of action is countered by concerns regarding toxicity.

Stem cell transplantation

The availability and safety of allogeneic SCT options have hugely increased with use of alternative donors, improved tissue-typing and volunteer unrelated donor availability and improvement in conditioning regimens (details covered in Chapter 37). Therefore, for the majority of young patients where curative intent is desired, SCT should be an option. However, while there is little doubt that allogeneic SCT is the most effective treatment modality to prevent leukaemic relapse in fit patients, the reduced risk of relapse gained with transplant has always to be balanced relative to SCT increased toxicity and risk of non-relapse mortality (NRM)/morbidity and to the likelihood of achieving remission with chemotherapy alone. Assessing this balance is currently difficult. Proper randomised trials to quantitate the relative benefit are generally lacking and trials that do involve transplant may be influenced by subjective selection biases regarding how truly comparable the comparator groups are. Moreover, many other variables such as the type of conditioning used, mutational profile and biology of disease and MRD status before transplant are also likely confounding factors. Many studies suggest that, in intermediate/adverse risk AML, relapse risk is on average reduced from ~45–50–~20–25% with transplant. However, partially offsetting this reduced risk, there is an increase in NRM and the possibility of graft versus host disease (GVHD) which is highly variable depending on patient fitness, donor source (blood versus bone marrow) and degree of match (fully versus partially matched), conditioning regimen and other factors such as CMV status, donor sex etc. This NRM risk is generally ~20% although higher levels might be acceptable if the relapse risk is particularly high. Based on these data, it is generally felt that proceeding with SCT in CR1 for favourable risk AML patients is not indicated, given their relapse risk is generally <40% with standard consolidation chemotherapy and they can generally be salvaged at relapse and offered a SCT in second CR (CR2). Conversely, in adverse risk disease, where relapse risk is generally >60%, but can be as high as 90% if MRD positive, the risk-benefit ratio in fit patients is generally in favour of proceeding with allogeneic SCT in most instances, even when NRM is >20%. AML patients within the intermediate-risk group represent the most difficult group to risk assess; however, recent evidence has demonstrated that MRD monitoring can provide objective guidance to risk-adapt for SCT therapy in this group, dependent on its presence or absence. Overall the expectation of cure for recipients of SCT is around 60% from the time of transplantation in intermediate/adverse risk AML patients although again this is modulated by several of the factors mentioned above. Although allogeneic SCT is the preferred option due to its additional graft versus leukaemia (GvL) effect, autologous SCT has been used for those patients lacking a donor and for consolidation in intermediate-risk disease with MRD negative CR, with equivalent OS to allogenic SCT, with the increased relapse rate balanced by the reduction in NRM to about 5–10%.

Maintenance therapy

AML therapy has historically consisted of blocks of chemotherapy with or without SCT consolidation followed by monitoring for relapse. This reflects the cumulative toxicity of standard chemotherapy and that, unlike other haematological

malignancies, evidence supporting the benefit of maintenance chemotherapy was lacking. The recent development of novel therapies such as FLT3 inhibitors or HMA, including oral preparations, may alter this paradigm given that these therapies have demonstrated efficacy as single agents, particularly in low disease bulk, and are less toxic than standard chemotherapy. The phase 3 QUAZAR study has recently reported that an oral formulation of azacitidine (CC-486, Onureg®), given as maintenance therapy in patients with intermediate- or high-risk AML >55 years in first remission after IC and not candidates for SCT improved median OS compared to placebo by 10 months with good quality of life, with this difference being maintained at last follow-up. Moreover, in a recent post-hoc analysis, Onureg improved survival independently of *NPM1* and *FLT3* mutational status at diagnosis and MRD status following IC, thus suggesting that its benefits are likely applicable to most AML subgroups. This treatment has now been approved by the FDA and EMA. FLT3 inhibitors have also been used as maintenance therapy based on their efficacy and relatively low toxicity. In the RATIFY trial patients in CR who did not proceed to SCT were given Midostaurin maintenance for 12 4-weeks cycles. This treatment was well tolerated but an unplanned post-hoc subset analysis was inconclusive with regards to the value of maintenance therapy. Nevertheless, Midostaurin maintenance is currently approved in patients not proceeding to SCT and routinely used in this setting. Post-SCT maintenance is also currently being investigated using similar approaches. So far, small studies in the post-transplant setting have failed to show convincing evidence of benefit from maintenance strategy using Azacitidine or Midostaurin, although the multikinase inhibitor Sorafenib was shown to reduce relapse risk in randomised trials post SCT in *FLT3*^{ITD} mutant patients. More recently however the randomised, double-blind MORPHO study has failed to show significant benefit in terms of relapse free survival for the more potent FLT3 inhibitor gilteritinib post SCT. However subgroup analysis of patients who had detectable MRD pre- or post-SCT showed benefit from gilteritinib maintenance. It does therefore appear that the benefit of maintenance therapy post-transplant is limited to specific subgroups, such as those with detectable MRD, and will likely be the subject of future studies.

Acute promyelocytic leukaemia

APML represents a special case, as the presence of the t(15;17)/*PML::RARA* translocation predicts sensitivity to treatment with all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO). APML is also particularly sensitive to anthracyclines and more recently has been shown to be highly sensitive to GO. The combination of ATRA and idarubicin is expected to provide survival rates in excess of 80%. Although maintenance therapy with courses of ATRA and orally available agents such as methotrexate and 6-mercaptopurine has been shown to provide additional benefit in some studies, the role of maintenance, particularly in patients who are molecularly negative after consolidation (currently >95% of cases) is in doubt. Moreover, a pivotal study from the Italian AML study group (GIMEMA) and the German–Austrian Acute Myeloid Leukaemia Study Group and Study Alliance Leukaemia in low- and intermediate-risk APML patients, i.e. those with presenting white cell count $<10\times10^9$/L, has demonstrated that the combination of ATRA and ATO alone can produce very impressive survival rates of over 95%. This has been confirmed in a similar UK NCRI trial which used less frequent arsenic dosing and included high-risk patients. These two trials have further changed the landscape of APML treatment and currently, the standard of care, at least for the 75% of low-risk patients, is a 'chemotherapy-free' combination of arsenic trioxide and ATRA. This will become even more attractive once oral formulations of arsenic become routinely available. One caveat with the 'chemotherapy-free' approach is the increased risk of differentiation syndrome, which is normally reduced by the inclusion of chemotherapy in regimens. This syndrome is accurately characterised by the presence of unexplained fever, weight gain, respiratory distress, interstitial pulmonary infiltrates, and pleural or pericardial effusion, with or without hyperleucocytosis. No single sign or symptom itself may be considered diagnostic of the syndrome, but at the signs of the earliest manifestations of suspected differentiation syndrome (e.g. unexplained respiratory distress), anti-leukaemic therapy should be interrupted and treatment with dexamethasone 10 mg i.v. 12-hourly should be initiated and continued until disappearance of symptoms and signs, and for a minimum of 3 days, given the fulminant nature of differentiation syndrome if left to progress. Although the chemotherapy-free approach is still not routinely used in high-risk patients (defined as those with presenting white counts $>10\times10^9$/L), it is however feasible if combined with a brief exposure to Gemtuzumab ozogamicin or idarubicin to reduce the risk of differentiation syndrome.

Previously, molecular monitoring in APML by RT-qPCR has provided a paradigm for molecular MRD monitoring in other AML subtypes. While the majority of patients (~95%) achieve molecular remission, approximately 10–25% will ultimately relapse following ATRA and anthracycline-based protocols. The majority of relapses can be predicted by re-emergence of *PML-RARA* transcripts detected in sequential bone marrow MRD monitoring samples, which are usually performed 3-monthly. However, as treatment protocols for APML have steadily improved, current consensus is that in patients with low-risk disease with rapid achievement of molecular remission, there is little benefit in post-therapy MRD monitoring. In high-risk patients, however, sequential MRD monitoring until 3 years post-treatment is recommended, due to the up to 25% risk of relapse and the ability of a rising MRD level to identify

impending overt relapse. This strategy therefore allows the institution of treatment intervention at the time of low disease bulk with the attendant reduced risk of bleeding and other complications compared to salvage at frank relapse.

Novel agents

Improved understanding of the molecular drivers and biology of AML has led to the development of multiple novel therapies. Most of these are currently in use in early phase trials for relapsed/refractory AML patient, although the most promising are now being also tested, often in combination with standard therapies, in untreated patients in phase 3 trials with the aim to improve outcomes in the diagnostic setting.

Epigenetic therapies

Aberrant transcription is a cardinal feature of AML and mutations that alter epigenetic regulators and transcription factors are highly recurrent in AML. Numerous epigenetic therapies have been developed and are currently being tested in AML. Amongst those more advanced in clinical development are the IDH1/2 inhibitors. IDH1/2 are metabolic enzymes recurrently mutated in 10–20% of AML patients. *IDH* mutations are gain-of-function mutations generating the 'oncometabolite' 2-hydroxyglutarate (2-HG), which inhibits multiple dioxygenase enzymes, including TET, Jumonji-C histone lysine demethylases and prolyl hydroxylase enzymes, resulting in increased DNA and histone methylation and aberrant transcription causing differentiation arrest. Inhibitors specific for the mutant proteins have been developed, which lead to a significant decrease in 2-HG levels and reverse the differentiation block in leukaemic blasts. Inhibitors of both IDH1 (Ivosidenib, Tibsovo™) and IDH2 (Enasidenib, Idhifa™) are now FDA-approved based on early phase trials results where they demonstrated relatively high remission rates in relapsed/refractory (R/R) *IDH*-mutant patients. Both inhibitors demonstrated good tolerability, although a specific side effect is the IDH-inhibitor-associated differentiation syndrome; similar in its clinical presentation to differentiation syndrome in APML following ATRA therapy and managed with corticosteroids and drug interruption. However, preliminary reports from a phase III study comparing Enasidenib to conventional care in R/R AML patients have failed to show a survival benefit (IDHENTIFY NCT02577406). Interest has now therefore shifted towards incorporating these inhibitors in combination therapies, the most advanced of which takes advantage of preclinical synergism between IDH inhibition and Azacitidine. Recently the AGILE trial, comparing the combination of Ivosidenib and Azacitidine versus Azacitidine alone in newly diagnosed *IDH1*-mutant AML patient unfit for IC has reported improved survival for the combination treatment (median overall 24.0 months with Ivosidenib and Azacitidine and 7.9 months with placebo and Azacitidine), leading to its FDA approval in this setting. However, this study has attracted some criticism as only two patients in the Azacitidine arm went on to receive Ivosidenib at relapse despite the latter being approved in the relapsed setting. Whether a sequential approach might be more tolerable in patients unfit for IC, who are often treated without curative intent, without jeopardising their survival rates remains to be proven. Interestingly with regards to this, the randomised phase 2 trial comparing Enasidenib plus Azacitidine versus Azacitidine alone in patients with newly diagnosed, mutant-*IDH2* AML has failed to show an improvement in survival, despite higher rates of response in the combination arm. This was due to the use of subsequent AML therapy in most patients randomly assigned to Azacitidine therapy, including Enasidenib in about a third of the patients. Finally, neither of the above trials used the Venetoclax/Azacitidine regime as a comparator, despite this being the current standard of care in this patient group. Given the evidence that *IDH*-mutant AML is particularly sensitive to Venetoclax/Azacitidine combinations, these trials have failed to clarify what is the best upfront therapy in *IDH*-mutant AML patients unfit for IC. Interestingly, given *IDH*-mutant AML sensitivity to Venetoclax-based therapies, early phase studies combining Venetoclax with either Enasidenib or Ivosidenib are currently ongoing, and it will be worth further considering if concomitant or sequential treatment with IDH inhibitors, Venetoclax and Azacitidine might further improve patient outcomes.

MLL-mutant AML has also been extensively studied as a subtype sensitive to epigenetic therapies. Amongst many partner proteins MLL-fusions requires the Menin co-factor to bind at critical genes and the DOT1L methyltransferase activation of these genes. DOT1L inhibition reduces the expression of critical MLL-fusion protein target genes, including the *HOXA* cluster and *MEIS1*, causing reduced proliferation and survival of AML cells, while its role in adult haematopoiesis is less critical thus providing a therapeutic window. Pinometostat (EPZ-5676) is the most clinically-advanced DOT1L inhibitor and has so far shown good tolerability with some remissions observed in early-phase trials in both paediatric and adult settings. Currently, combination trials with both hypomethylating agents and standard cytotoxic chemotherapy are underway. The main limitation of Pinometostat is its poor pharmacokinetics, requiring continuous infusion of up to 28 days. Novel orally available DOT1L inhibitors with improved pharmacokinetics are currently being developed.

The adaptor protein Menin binds to both the N-terminus of wild-type MLL and MLL-fusion proteins and is required for aberrant gene expression. Preliminary data from the ongoing trial of KO-539 (KOMET-001 NCT04067336), which acts by

disrupting the Menin-MLL interaction, has shown evidence of remarkable efficacy, even at low doses, in R/R disease regardless of mutational profile. In addition, a recently reported trial of the compound SNDX-5613 (Revumenib) in the relapse refractory setting for predominantly MLL1/KMT2A-r or NPM1c- mutated AML (AUGMENT-101 NCT04065399), has documented an excellent 53% combined response rate, with an overall CR/CRh rate of 30%.

Therapies targeting specific transcription factors

TP53 is a transcription factor with multiple roles in regulating cell cycle, apoptosis, DNA repair and metabolism and is recurrently mutated in ~10% of AML. *TP53* mutations are enriched in therapy-related AML and are associated with a particularly dismal prognosis, predicting poor response to standard cytotoxic chemotherapy. Eprenetapopt (APR-246) is a small molecule that selectively induces apoptosis in *TP53*-mutant cancer cells, through thermodynamic stabilisation of the p53 protein and shifting it towards a functional conformation. Promising Phase I studies with eprenetapopt in AML patients have demonstrated clinical activity and reactivation of p53-dependent pathways. Recently a phase 1/2 trial assessing the combination of Azacitidine and Eprenetapopt specifically in *TP53*-mutant myeloid malignancies has reported very promising response rates of 44% with median survival in excess of 10 months which compares positively with historical controls in this high-risk group. Eprenetapopt has now been granted breakthrough therapy designation for the treatment of patients with *TP53*-mutant myeloid malignancies by the FDA, in combination with Azacitidine.

Immunological therapies

Antibody treatment

The success achieved through allogeneic SCT in curing AML is proof of its sensitivity to cellular immunological therapies. Moreover, the efficacy of GO also suggests sensitivity of AML to antibody therapies. Despite this, immunological therapies targeting AML have generally lagged well behind those targeting lymphoid malignancies. However, recently, several novel antibody therapies have emerged targeting either immune checkpoint proteins (CD47) or Tumour Necrosis Factor (TNF) receptor ligand (CD70). These are currently at different stages of development with variable efficacy results and will likely require ongoing optimisation to fulfil their potential. Bispecific T-Cell engagers (BiTEs) or Dual Affinity Retargeting Proteins (DARTs) are novel immunological therapies that bring together effector cells (T-cells) with target cells expressing specific markers. Blinatumomab (CD3xCD19 BiTE) is a paradigmatic example of this concept and is currently licensed for Acute Lymphoblastic Leukaemia therapy. Similar DARTs targeting AML antigens such as CD123 and CD33 are also being developed with results regarding their efficacy and tolerability awaited.

Cellular immunotherapies

Chimeric antigen receptor T-cells (CAR-T) and T-cell receptor rearranged donor T-cells, are also starting to find their niche in AML therapy. They already have a well-established role in lymphoid malignancies; however, their development in AML has been hindered by the lack of a truly AML-specific target. CD33, CD123 and CLL-1 are currently all being studied as promising targets but their expression on normal HSC has thus far limited their utility. This, together with challenges in manufacturing CAR-T cells for AML patients in a timely fashion and the immunosuppressive environment created by AML, have proven the main difficulties. The incorporation of 'safety switches' to modulate activity of the CAR-T cells, identification of leukaemia neoantigens amongst the targets and use of alternative effector cells, such allogeneic modified T or NK cells, are currently being used as possible solutions. Finally enhancing immune responses via the use of immune checkpoint inhibitors or other small molecule inhibitors affecting immune milieu will likely overcome the immunosuppressive environment generated by AML and increase the efficacy of cellular therapies.

For all novel therapies, it is important to note that given their targeted nature, and often poor cytotoxicity in comparison to standard chemotherapy, assessing their responses as single agents predominantly in terms of remission rates in frank relapsed/refractory disease might be inappropriate. The experience with GO has clearly shown that novel therapies that do not affect remission rates can still significantly improve patient OS when used in combination. Novel methods of assessing response are therefore required, perhaps based on predictive biomarkers and early use in rational combinations is also to be encouraged. Finally, the identification of AML genotypes sensitive to specific agents is necessary, so that their use is limited to the patients most likely to benefit from them, thus moving AML therapy towards a more personalised, precision medicine approach.

Resistance and relapse

With current intensive therapeutic approaches over 80% of young (<60) AML patients will enter complete remission, dropping to around 60% in the >60s. Thus, a sizeable proportion of patients are refractory to current induction AML therapy. Moreover, of those entering remission, around 50% or more will relapse. Unfortunately, the choice of therapy in this setting cannot be guided by strong evidence, as most of the clinical studies

on relapse are single-arm. The fundamental decision when managing patients with refractory or relapsed disease is to establish clearly the aim of therapy, i.e. whether attempting curative therapy or adopting a palliative approach, aiming to achieve a degree of disease control while optimising quality of life. Reinduction chemotherapy is unlikely to provide durable disease control even in favourable-risk patients. It is, therefore, advisable whenever possible to consolidate a second remission with allogeneic SCT, if feasible, as this has been shown to be associated with improved long-term survival. If not feasible, an autologous transplant might provide a valid alternative, particularly if MRD-negative remission is achieved. Consolidation with a second allogeneic SCT can also be used in patients who have already received one in first remission, however for those relapsing within 6 months from the first transplant alternative approaches are required.

Whether the advent of novel therapeutics will change the outlook for refractory/relapsed disease remains to be seen. Certainly, some of the novel therapies discussed above have shown, albeit in single-arm studies, very promising response rates with improved tolerability compared to standard chemotherapy, and at least for the FLT3 inhibitor Gilteritinib, there is phase 3 trial evidence of its superiority compared to standard therapies in *FLT3*-mutant patients with R/R disease. It is conceivable that novel therapies, including those already approved for frontline therapy such as Venetoclax, might be better tolerated and a few retrospective studies have shown the activity of Venetoclax-based therapy at relapse in a proportion of AML patients. As a result, an increased percentage of patients could be bridged to transplant and would therefore be eligible to be treated with curative intent at relapse. Finally, it is hoped that the usage of novel therapies upfront at diagnosis may decrease the likelihood of R/R disease, thus reducing the scale of the problem.

APML is again a special case. In patients with suspected relapse of APML, it is important to confirm *PML::RARA* positivity, to exclude the possibility of therapy-related MDS/AML and to identify patients likely to benefit from molecularly targeted therapy. Patients can respond again to retinoic acid and chemotherapy, but recently arsenic trioxide and the CD33-directed immunoconjugate GO have been found to be effective. Remission rates above 80% have been reported; interestingly, following consolidation therapy, a similar proportion can be returned to RT-qPCR negativity. This permits the opportunity for autologous transplantation, the successful outcome of which depends on the graft and, preferably, the patient being molecularly negative. For patients who remain molecularly positive following salvage, allogeneic SCT is indicated. Arsenic trioxide is now the standard first-line salvage approach in *PML::RARA+ APML*; indeed some relapsed patients can be cured with ATRA + ATO protocols, raising questions as to whether consolidation with an autograft is necessary in all patients who go back into molecular remission.

Future directions

Classification and risk stratification

The last decade has seen an unprecedented progress in the classification and risk stratification of AML patients mostly driven by our improved ability to detect somatic mutations and link these to patterns of clinical outcome. It is plausible that in the next decade, this will be further refined through the application of epigenetic, metabolic and proteomic characterisation of AML samples. These multi-omic approaches will help improve disease classification, identify predictive biomarkers of response and move towards a more personalised approach to AML therapy. It is also likely that single-cell approaches, as already shown for single-cell genomics, will allow further understanding of disease heterogeneity within patients which might have implications for defining the best combination therapies for each patient. All these data will be then leveraged through knowledge banks algorithms that can help devising personally tailored therapeutic decisions. This will require significant investments to remain up to date as the therapeutic armamentarium expands and molecular understanding of AML deepens and will also face significant logistical and regulatory hurdles.

Therapy

The advantages of a deeper understanding of the molecular and biological features of AML will remain untapped unless novel therapies are devised and tested within large clinical trials to assess their efficacy and the most rational way to deploy them. Future trials will have to incorporate the knowledge generated via different approaches from the diagnostic material to guide therapy choices while adapting these choices in *real-time* based on further assessment of response (MRD) and changing disease biology at different time points along the therapeutic journey of each patient. While such a precision medicine approach is eminently feasible, as already shown by the Leukaemia and Lymphoma Society-sponsored 'Beat AML' trial, the increasing number of new treatments available will demand novel approaches to clinical trial design to ensure more rapid progress. This, in turn, will require much greater international collaboration to provide sufficient numbers of the patient subgroups and at different stages of disease (presentation, relapse, etc.) and different statistical methods to allow us to draw conclusions from these studies. It is also possible that in the future, if we can fully understand the trajectories of AML development, we may be able to intervene earlier, at the stage of CH or of an antecedent myeloid neoplasm, such as an MPN or MDS and prevent or delay beyond the human lifespan the development of AML. We are living through extraordinary times in the field of AML biology and therapy but will need to devise creative solutions to fully translate the benefit of these technological and knowledge-based advancements into improved patient care and outcomes.

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CHAPTER 33

Chronic myeloid leukaemia

33

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Introduction

Chronic myeloid leukaemia (chronic granulocytic leukaemia) is a myeloproliferative neoplasm characterised by rearrangement of the long arms of chromosome 9 and 22, resulting in the Philadelphia (Ph) chromosome, creating the fusion oncogene *BCR::ABL1*. This genetic event that encodes for a constitutively active tyrosine kinase occurs in a haemopoietic progenitor and confers proliferative and antiapoptotic effects. The natural history of untreated CML is triphasic. It most commonly presents in chronic phase (CP), characterised by markedly increased myeloid activity, resulting in increased numbers of morphologically and functionally normal granulocytes, predominantly comprised of neutrophils and myelocytes. Basophilia is a distinct feature of CML, although eosinophilia is also usually present. Thrombocytosis is commonly seen. Without effective treatment, CP-CML invariably progress over 5–10 years, with rising blast percentage, cumulating in marrow failure and cytopenia. Historically, a transitory, accelerated phase (AP) may precede blast phase (BP) disease, though the most recent WHO classification no longer recognise CML-AP as a distinct identity. In CML-BP (blast crisis or blastic transformation), the disease clinically resembles acute leukaemia and the outcome is dismal. The risk of disease progression has been dramatically reduced in the era of tyrosine kinase inhibitor (TKI) therapy. Although therapy is expected to be lifelong in many, a significant proportion of patients who achieve deep molecular remission are able to cease their TKI therapy entirely without losing their remission status, and treatment-free remission is now an accepted long-term goal of therapy for most patients.

Epidemiology and aetiology

The incidence of CML in developed nations has been relatively stable over the last decade and remains at 1–2:100,000, with a slight male predominance. The disease is most commonly diagnosed in the fifth and sixth decades of life in developed countries. In the developing world, presentations in the third and fourth decades are more common, possibly reflecting younger population demographics. CML is rare in the paediatric population.

Apart from exposure to ionising radiation (evidence derived from epidemiological studies of Hiroshima and Nagasaki atomic bomb survivors), there is no other known risk factor leading to the development of CML, whether inherited or acquired.

Clinical features, natural history, laboratory haematology and cytogenetics

The laboratory features of CML in its various phases, as defined in the two most commonly used classifications, are summarised in Table 33.1. The European Leukaemia Net (ELN)/MD Anderson Cancer Centre criteria were used to define CP in many pivotal TKI studies and still have relevance for interpretation of clinical data. The WHO classification is increasingly gaining acceptance; its 2022 revision no longer recognises a separate disease entity of accelerated phase CML, placing more emphasis on high-risk features associated with progression to blast phase from chronic phase. An important distinction between the two is the higher myeloblast count for

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 33.1 Definition of advanced phase CML disease.

	ELN	WHO
Accelerated phase	PB or BM blast 15–29% PB basophils ≥20% PB or BM blast + promyelocytes ≥30% Clonal evolution Platelets ≤100 × 109/L unrelated to therapy	The WHO (2022) no longer recognises accelerated phase CML as a separate entity
Blast crisis	PB or BM blast ≥30% Extramedullary blast proliferation, apart from spleen	PB or BM blast 20% or more Extramedullary blast proliferation The presence of increased lymphoblasts in peripheral blood or bone marrow.

BM, bone marrow; PB, peripheral blood.

CML-BP, set by the ELN (30% rather than 20% used by WHO), and a role for excess promyelocytes in the determination of CML-AP (Table 33.1).

Chronic phase

The majority of patients (around 95%) are diagnosed with CML-CP. Many are asymptomatic and the diagnosis is often incidental to investigation for an unrelated medical problem. The most common presentations include unintentional weight loss, night sweats, fatigue and splenomegaly (which may cause early satiety and anorexia). The most striking haematological abnormality is usually a leucocytosis, with the differential heavily favouring mature neutrophils, band forms and myelocytes. Eosinophilia is common, and absolute basophilia is usually present (Figure 33.1a). Absolute monocytosis is uncommon and, if present, usually associates with the e1a2 breakpoint (see below). Rarely, patients may present with a marked thrombocytosis with only mild leucocytosis. Unlike other myeloproliferative disorders, CML is rarely associated with arterial or venous thromboses. Dysplasia is uncommon in CML, and if present, should prompt consideration of atypical CML (MDS/MPN with neutrophilia in WHO 2022) as a differential diagnosis.

The CML-CP marrow is usually grossly hypercellular as a result of markedly increased myeloid activity (Figure 33.1b). Myeloid maturation is normal or slightly left-shifted, and blasts are usually less than 5%. The myeloid-to-erythroid ratio is markedly increased and megakaryocyte numbers may be either decreased or increased compared to the normal marrow; small, hypolobated forms are common. Increased reticulin fibres are seen in 30% of cases and correlate with larger spleen size. Pseudo-Gaucher cells and sea blue histiocytes may be seen secondary to increased numbers of phagocytic cells amidst increased cell turnover.

Accelerated phase (AP)

While CML-CP is characterised by over-production of myeloid cells, essentially with normal morphology and function, AP disease is characterised by either impaired differentiation or drug resistance. There may be an increased myeloblast percentage in peripheral blood or marrow, as well as increasing leucocytosis, splenomegaly, basophilia or thrombocytosis, despite ongoing therapy. Thrombocytopenia, unrelated to therapy, may herald progressive marrow failure. Cytogenetic abnormalities additional to those found in the baseline Ph^+ clone are often present at progression to AP, a phenomenon termed clonal evolution. These abnormalities frequently include duplication of the Ph chromosome, trisomy 8 or 19 and isochromosome 17q. CML-AP usually progresses to CML-BP within 1–2 years without appropriate treatment. In its 2022 revision, the WHO no longer recognises CML-AP as a separate disease entity, though this term continues to be used clinically, especially in the context of historical data.

Blastic phase (BP)

CML-BP is characterised by rapidly rising blast counts and uninhibited growth of myeloid precursors with absence of maturation or differentiation, resulting in marrow failure and cytopenia. Morphologically, CML-BP is indistinguishable from other cases of acute leukaemia. In ~70% of cases, the blasts are of myeloid lineage by morphology and immunophenotype, and may express antigens associated with granulocytic, monocytic, megakaryocytic or erythroid differentiation. Aberrant expression of lymphoid markers (e.g. CD7) is common. Blasts in the remaining 25–30% of cases usually express lymphoid antigens of B lineage. Aberrant expression of myeloid antigens in CML lymphoid BP is also quite common.

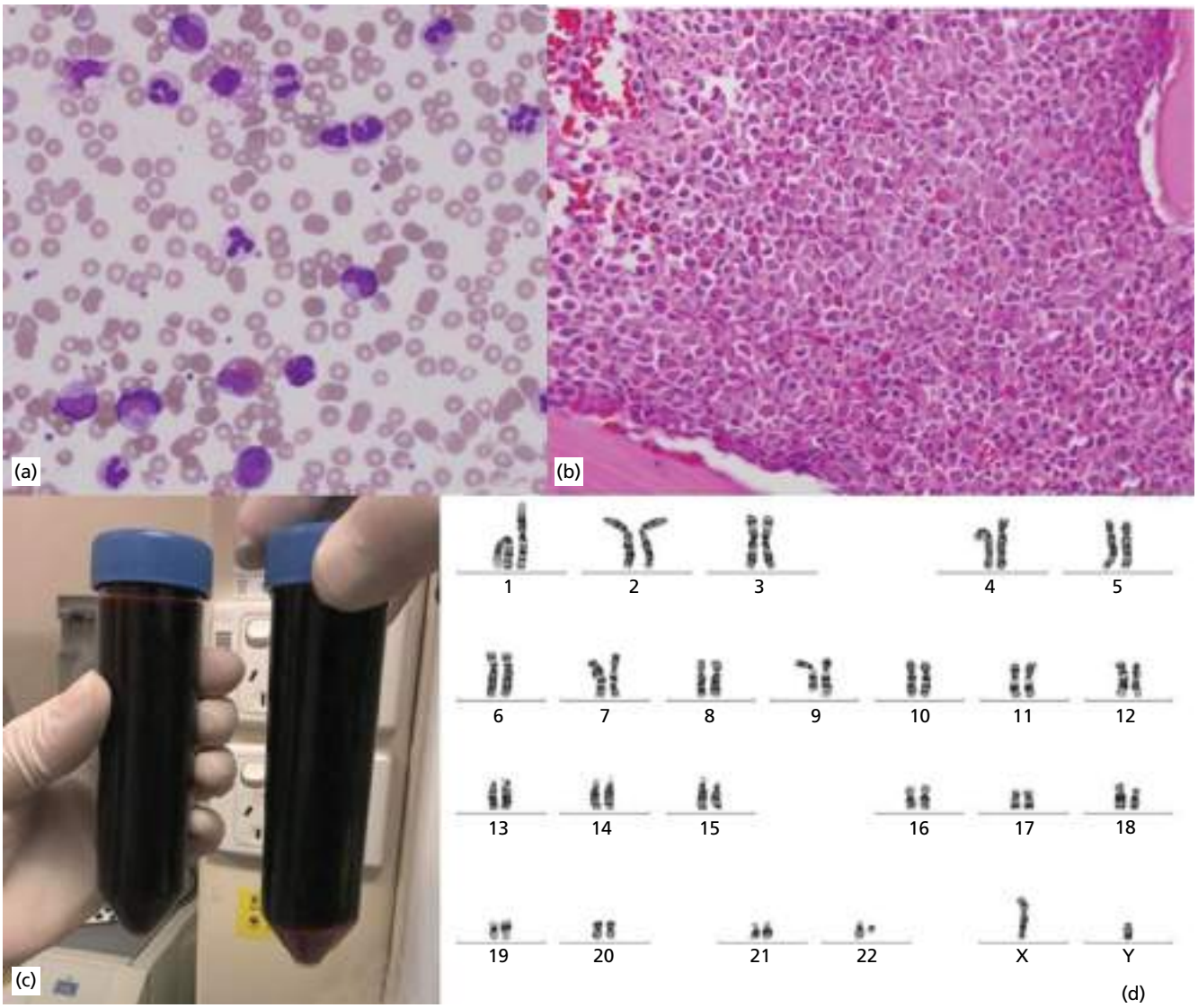


Figure 33.1 (a) Blood film from a newly diagnosed CML-CP patient aged 47 years. Leucocytosis is prominent, with increased neutrophils and myelocytes, but no obvious dysplastic features. Several myelocytes, a basophil and an eosinophil can be seen towards the bottom of the photomicrograph. (b) A grossly hyper-cellular marrow trephine from the same patient. (c) Whole blood is processed prior to RQ-PCR. Red cell lysis agent is added to whole blood and a white cell pellet is obtained after centrifugation. On the right, a newly diagnosed CML patient yielded a sizeable white cell pellet at the bottom of the tube, compared to that of a normal control on the left. (d) Karyogram showing the translocation t(9;22) (Source: (d) Ms. Sarah Moore, SA Pathology, Adelaide, Australia. Reproduced with permission.)

The WHO 2022 also recognises t(9;22) as a defining genetic abnormality in de novo AML, although distinguishing these cases from patients presenting in myeloid blast crisis of CML may be challenging.

Cytogenetics and molecular biology

The diagnosis of CML rests on demonstration of the Ph chromosome with cytogenetic analysis, or the *BCR::ABL1* fusion gene with RT-QPCR (reverse transcriptase quantitative polymerase chain reaction, also known as RQ-PCR, qRT-PCR or QPCR), in the context of compatible blood and marrow morphology. Cytogenetics and RQ-PCR offer complementary information and both tests should be performed routinely at diagnosis.

The Ph chromosome was first linked to CML in 1960 by Peter Nowell and David Hungerford in Philadelphia. Janet Rowley in 1973 showed this to be a result of a reciprocal translocation between the long arms of chromosome 9 and 22 (Figure 33.1d).

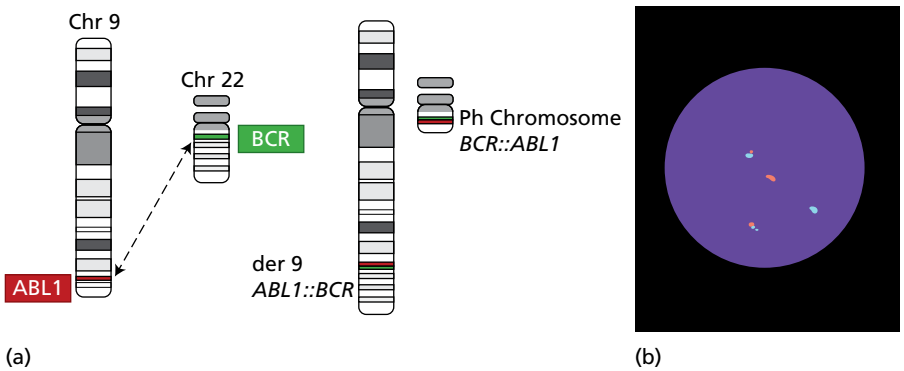


Figure 33.2 (a) The t(9;22) translocation leads to juxtaposition of genetic material from chromosomes 9 and 22, with formation of two fusion genes *BCR::ABL1* and *ABL1::BCR*, the former being crucial to the pathogenesis of CML and Ph+ ALL. (b) Fluorescence *in-situ* localisation. Hybridisation of an oligonucleotide sequence complementary to the *BCR* gene attached to a fluorophore (green in this case) leads to its localisation in a metaphase spread. The same can be done for *ABL1* (red in this case). A normal cell has two red and two green signals. A t(9;22) translocation event will juxtapose red and green fluorophores (with some probe sets seen as two fused yellow signals), one from *BCR::ABL1* and one from *ABL::BCR (*the two signals to the left of the picture). The remaining red and green signals originate from the normal chromosome, which Is not rearranged (Source: (b) Ms. Sarah Moore, SA Pathology, Adelaide, Australia. Reproduced with permission. FISH probe set from Abbott Diagnostics, Vysis LSI BCR/ABL Dual Colour, Dual Fusion Translocation Probe.)

This event, now denoted as t(9;22)(q34;q11), is known to fuse the *BCR* (breakpoint cluster region) and *ABL1* genes together. The resulting *BCR::ABL1* fusion gene encodes a constitutively active tyrosine kinase that is central to the pathogenesis of Ph^+ leukaemias (Figure 33.2a). Variant translocations, where *BCR::ABL1* resides on a third chromosome, is seen in ~5–10% of patients and may be cryptic, discernible only with fluorescent *in situ* hybridisation (FISH) analysis (Figure 33.2b). Amplification of *BCR::ABL1* can result from duplication of the Ph chromosome, which, along with trisomy 8, i(17q) and trisomy 19, leads to increased risk of blastic transformation, and together these are referred to as major route cytogenetic lesions. Chromosome 3 abnormalities, and in particular changes involving the 3q26.2 locus where the *EVI* gene resides, are also associated with disease resistance. Cytogenetic analysis at diagnosis, therefore, remains important for prognostication as these changes are not discernible on FISH or RQ-PCR. Apart from CML, t(9;22) is also seen in a significant proportion of adult *de novo* high-risk acute lymphoblastic leukaemia cases. The finding of t(9;22) in *de novo* AML is rare. Although recognised in the current WHO classification, it is challenging to distinguish this from an undiagnosed antecedent CML-CP.

The breakpoints of the *BCR* and *ABL1* genes usually occur within the introns of each gene and are unique to each individual (Figure 33.3). The *ABL1* breakpoint is almost invariably located in the intron between exons 1a/1b and 2, whereas the *BCR* breakpoint usually occurs in an intron between exons 13–15. The resulting fusion transcripts are named e13a2 and e14a2 transcripts (previously known as b2a2 and b3a2); both may coexist in one individual due to alternate splicing. These transcripts encode for a 210 kDa protein product (p210), seen in the majority of CML patients. A breakpoint immediately downstream of the first *BCR* exon produces the e1a2 transcript, which encodes the p190 *BCR::ABL1* protein. This is more commonly seen in Ph^+ ALL and is infrequent in CML. Rarely, the *BCR* breakpoint occurs between exons 17–20, leading to the larger p230 protein from the e19a2 transcript, often associated with a more prominent neutrophilia and thrombocytosis. Other transcripts such as e6a2, e8a2 and e18a2 are also occasionally reported.

The translocation is carried by the leukaemic stem cell population, such that both myeloid and lymphoid progeny may carry t(9;22) in an affected individual. ABL1 is a tyrosine kinase with multiple substrates. Its activity is tightly regulated by the phosphorylation status of critical tyrosine residues on ABL1 itself. Juxtaposition of BCR and ABL1 promotes dimerisation of *BCR::ABL1* proteins through a coiled-coil domain in BCR and formation of tetramers through subsequent dimerisation of dimers (Figure 33.4). Critical tyrosine residues are brought into close proximity with adjacent ABL1 kinase domains, leading to autophosphorylation and activation. In addition, native ABL1 is usually regulated by autoinhibition mediated by intramolecular binding of an N-terminus myristate moiety into the kinase domain. This myristate moiety is lost through the translocation event. These modifications lead to constitutive activation of ABL1, which through downstream messengers, results in cellular effects such as loss of growth inhibition,

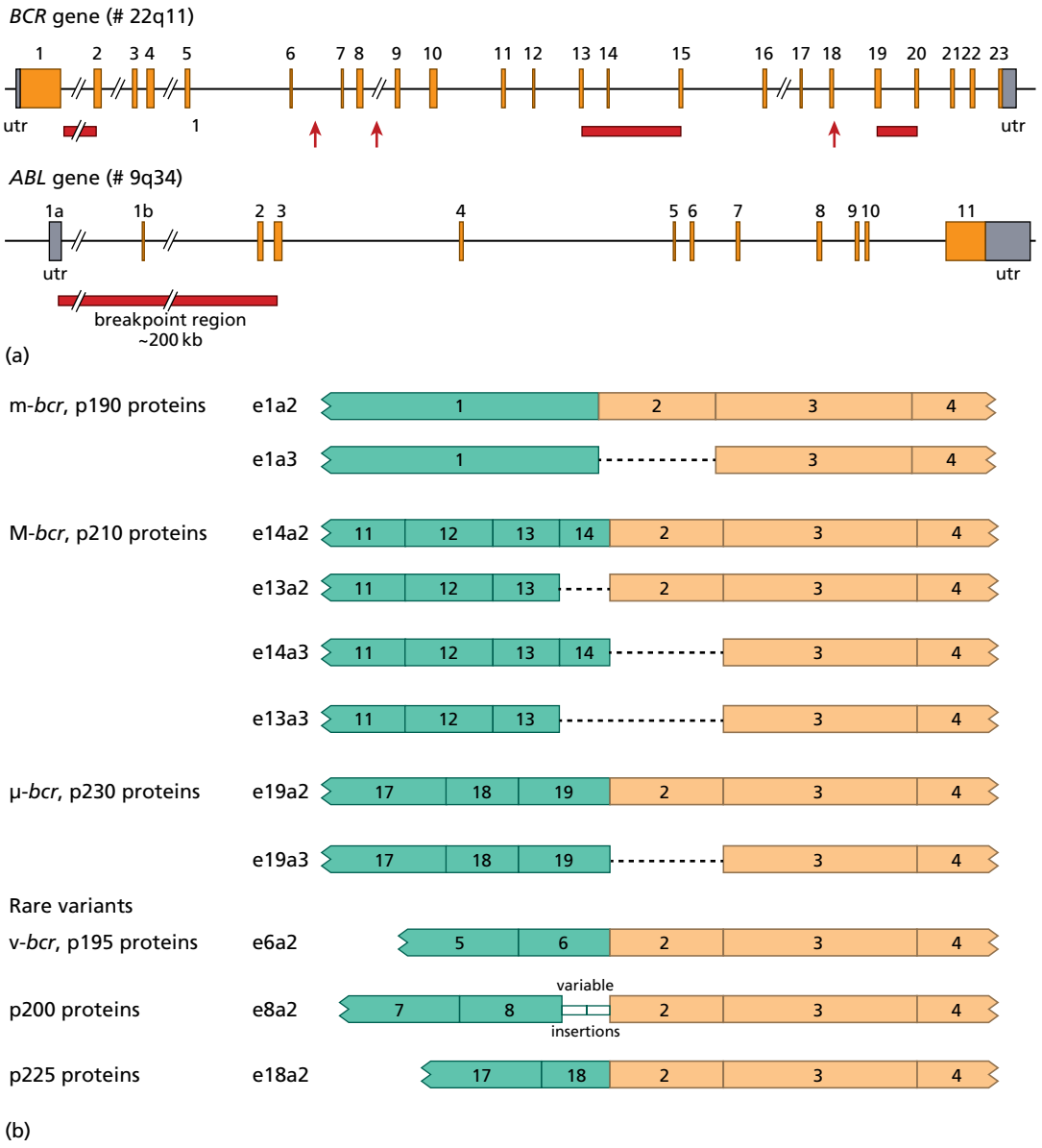


Figure 33.3 (a and b) Formation of the *BCR::ABL1* fusion gene. Breakpoint in the *ABL1* gene almost invariably occurs in the intron between exons 1–2. In contrast, breakpoints in the *BCR* gene may occur in introns between exons 1–2, 13–15 and 19–20. These regions are also known as the m-*BCR,* M-*BCR* and μ-*BCR* regions, respectively, and in turn yield the p190, p210 and p230 *BCR::ABL1* proteins. The e13a2 and e14a2 (also known as b2a2 and b3a2 respectively) transcripts are most commonly encountered in CML, whereas Ph+ ALL cases are usually associated with the e1a2 transcript. The e13a2 and e14a2 transcripts may coexist in the same patient secondary to alternative splicing in patients with the genomic e14a2 breakpoint. Other breakpoints (v-*BCR*) are also occasionally encountered (Source: Weerkamp *et al.* (2009) *Leukemia*. **23**: 1106–17. Reproduced with permission of Nature Publishing.)

decreased apoptosis and decreased adherence to bone marrow stromal cells. Many of the downstream pathways are well-studied and involve proteins including RAS/RAF/MEK/ERK, PI3K/AKT, STAT and MYC. *BCR::ABL1* also leads to genomic instability and facilitates disease transformation through accumulation of subsequent genetic lesions. DNA damage, such as double-stranded breaks, are mediated through increased ROS (reactive oxygen species) generated by *BCR::ABL1*. The repair mechanisms secondary to ROS-associated DNA damage in *BCR::ABL1* positive cells are more error-prone compared to normal mammalian cells, which in turn contributes to genomic instability.

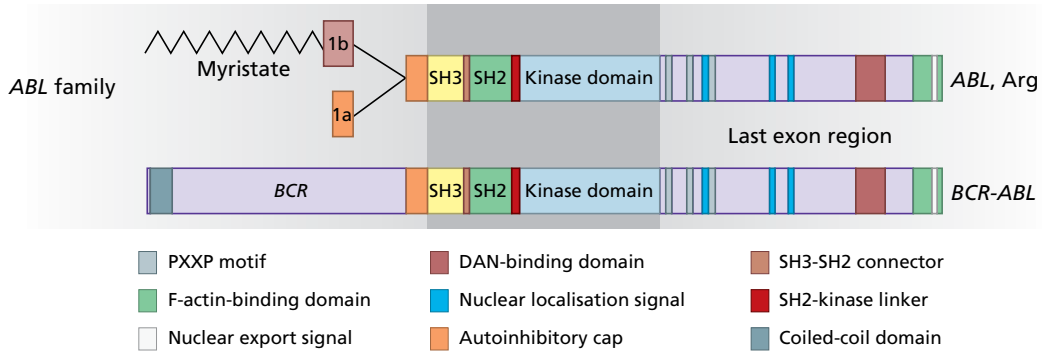


Figure 33.4 The native ABL1 protein possesses either an auto-inhibitory cap region encoded by exon 1a, or a myristate moiety encoded by exon 1b. The myristate moiety can fold over and bind to a pocket on the same ABL1 molecule to exert an auto-inhibitory effect. Replacement of exon 1a/b with BCR not only leads to loss of auto-inhibition, but also leads to tetramerisation through introduction of the coiled-coil domain in *BCR*, resulting in cross phosphorylation of critical tyrosine residues on neighbouring ABL1 and constitutive activation (Source: Hantschel and Superti-Furga (2004) *Nat. Rev. Mol. Cell. Biol.* **5**: 33–44). Reproduced with permission of Nature.)

Measurement of residual disease and monitoring with cytogenetics and *BCR::ABL1* RQ-PCR

CML patients receiving therapy are commonly monitored with blood counts, marrow cytogenetics and molecular assays. A complete haematological response (CHR), the first treatment milestone, is achieved with normalisation of blood counts and morphology, as well as resolution of splenomegaly. Achieving CHR from treatment with cytotoxic agents (such as hydroxycarbamide) has little or no impact on the risk of transformation and can only be regarded as a short-term goal of therapy or palliative treatment.

When patients are successfully treated with tyrosine kinase inhibitors that specifically target the Ph^+ clone, the risk of transformation starts to fall when a reduction in the overall number of leukaemic cells can be achieved. This can be assessed through bone marrow cytogenetics, or Fluorescent *in situ* Hybridisation (FISH). However, assessment of RQ-PCR for *BCR::ABL1* in the peripheral blood is a more sensitive disease monitoring tool. Due to its demonstrated clinical significance correlated with specific transcript levels, and the ease of use, RQ-PCR has gained wide acceptance. *BCR::ABL1* RNA transcripts, measured from a peripheral blood sample, correlates well with the number of residual Ph^+ cells on marrow cytogenetics. The transcript numbers of a control gene such as *BCR, ABL1* or *GUSB* are usually measured from the same sample. The test result is reported as a ratio of *BCR::ABL1* transcripts numbers to control gene transcript numbers, as a percentage. As RNA is labile, control gene transcript numbers differentiate acceptable specimens from those subjected to degradation from transport and storage.

Reporting results on the International Scale (IS) allow inter-patient and intra-patient comparability between different laboratories through the use of a simple conversion factor. By convention, 100% IS is a diagnostic baseline value derived from 30 diagnostic samples from the IRIS (International Randomised Study of Interferon and STI571) study. As well as a ratio, it is also common to describe the residual tumour burden as a $\log_{10}$ reduction from the standardised baseline, with 10, 1, 0.1% being a MR1, MR2 and MR3 log disease reduction, respectively (Figure 33.5). A MR2 response, or *BCR::ABL1* of ≤1% IS, is a convenient and reliable

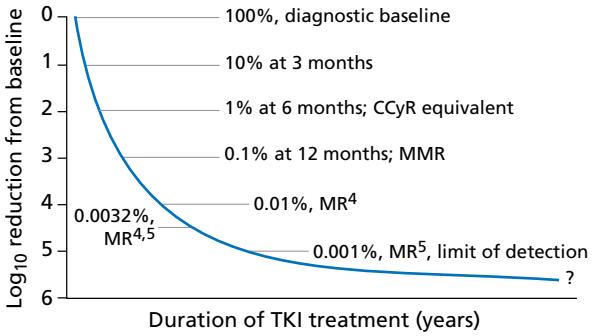


Figure 33.5 Idealised optimal reduction in *BCR::ABL1* over duration of TKI treatment. A RQ-PCR value of 100% on the international scale is derived from the baseline value of a subgroup of patients in the IRIS study. After 3 months of TKI treatment, patients should have a *BCR::ABL1* value below 10% (termed early molecular response – EMR). After 6 months, patients should achieve *BCR::ABL1* ≤1%, which is a surrogate for CCyR. After 12 months of therapy, the optimal response is considered MMR or *BCR::ABL1* ≤0.1%. Deeper molecular responses may follow after a variable period. MR^5 or $MR^{4.5}$ act as a platform for patients who wish to be considered for an attempt at treatment-free remission. RQ-PCR assays or digital PCR assays with sensitivity to >5 logs below the diagnostic baseline are not routinely available but are actively being studied in the research setting.

surrogate for CCyR. MR3, or *BCR::ABL1* of ≤0.1% IS, is a milestone response also referred to as major molecular response (MMR, orMMolR), associated with significantly superior event-free survival. Deeper responses, such as MR^4, $MR^{4.5}$ and MR^5 have also recently been defined as equivalent to *BCR::ABL1* of ≤0.01% IS, 0.0032% IS and 0.001% IS, respectively.

In general, patients receiving TKI therapy should have RQ-PCR testing every 3 months for the first 2 years, then every 3–6 months if MMR is achieved. Testing RQ-PCR monthly within the first 3 months of starting therapy may give an additional indication of how sensitive the disease is to treatment. This may be calculated as the halving-time – the number of days it takes for the *BCR::ABL1* to reduce by 50%, with shorter halving-time indicating more sensitive disease. Patients who fail to achieve time-dependent treatment targets (see later) may benefit from more frequent testing. An increase of RQ-PCR value of more than fivefold, or confirmed loss of previously achieved milestones, may be a consequence of treatment resistance and is worthy of further investigation.

Treatment

Oral cytotoxics, such as hydroxycarbamide (hydroxyurea), are used commonly for cytoreduction of leucocytosis before confirmation of diagnosis (leukapheresis can also be used in more extreme cases where symptoms or signs suggesting leucocytosis are present). During the initial cytoreduction phase, it is common to co-administer allopurinol to blunt any rise in serum urate and to monitor for electrolyte disturbances carefully.

Once diagnosis is confirmed, definitive treatment with a TKI should commence. Historical treatments such as oral busulfan have no role in the current treatment landscape. Although interferon-α was the gold standard treatment in the pre-TKI era and can induce durable complete cytogenetic response in ~20% of patients, it is now infrequently prescribed due to its toxicity profile, limiting generalised use except in pregnancy where TKIs must be avoided. However, pegylated interferon, a modified interferon that has slower clearance and a more favourable toxicity and efficacy profile, has resulted in a reemergence of its use. The role of pegylated interferon as a low-dose adjunct to TKIs is currently being examined in several clinical studies.

TKI treatment has transformed a previously fatal disease for many into a manageable chronic illness with excellent long-term survival and is the standard of care in CML-CP and AP. A recent Swedish population-based study suggested that the years of life lost attributable to a diagnosis of CML-CP in the TKI era to be minimal, observing a similar life expectancy in CML patients as compared with aged-matched population controls. Patients with CML-BP may be treated either with a TKI alone or in combination with chemotherapy, though duration of response tends to be short without consolidation with an allogeneic stem cell transplant.

The characteristics of the five most commonly used TKIs are discussed below. Imatinib was the first TKI to be approved for the treatment of CML and Ph+ ALL. It was followed by the discovery of second-generation TKIs such as nilotinib, dasatinib and bosutinib, and subsequently the third-generation TKI ponatinib. TKIs inhibit the kinase activity of *BCR::ABL1* through their binding to the ATP-binding pocket. A more recently discovered class of ABL inhibitors called STAMP – Specifically Targeting the ABL Myristoyl Pocket – shows therapeutic promise and will be discussed later in the chapter. The lead compound in the class, asciminib, has recently been approved for use in CML-CP patients with resistance or intolerance to 2 or more ATP pocket-binding TKIs.

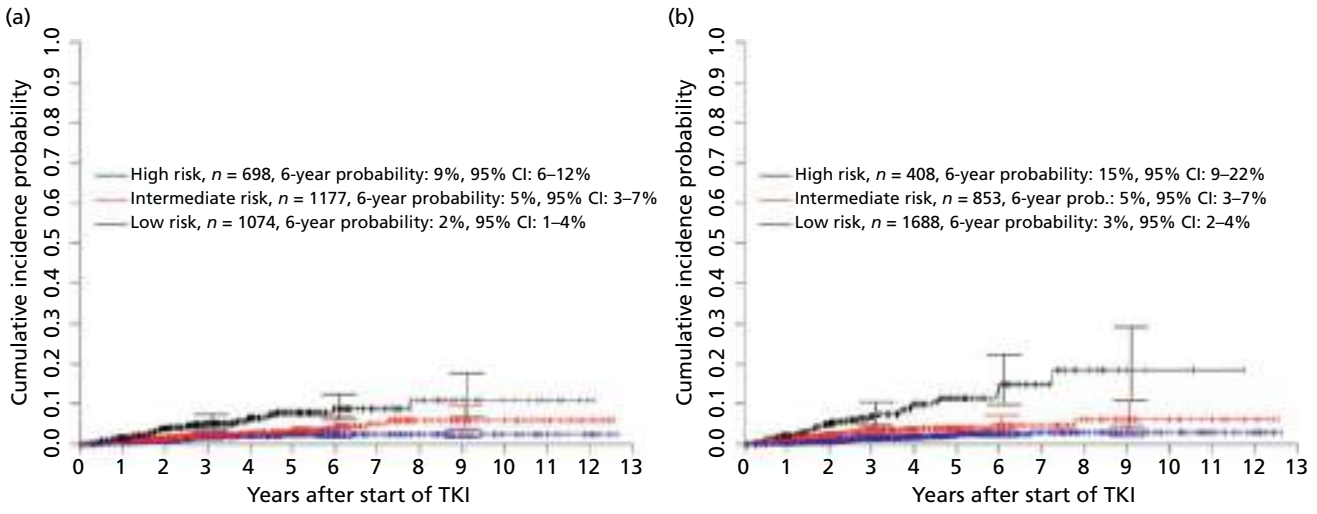
Haematological toxicities (anaemia, thrombocytopenia and/or neutropenia) are commonly encountered in the first few months of TKI treatment and usually improve with time as repopulation of the marrow by normal Ph-negative clones occurs. Dose intensity, especially in the initial months of treatment, is critical to the long-term success of TKI treatment. Supportive measures, such as colony growth-stimulating factor (GCSF, EPO) or transfusion support may be used as temporary adjuncts to allow for ongoing TKI treatment. Screening for past or chronic hepatitis B virus (HBV) infection is recommended, as HBV reactivation associated with TKI treatment resulting in fulminant hepatitis had been reported.

Assessment of CML risk score

The likelihood of a patient achieving the desired therapeutic outcome with TKI therapy may be predicted using clinical scoring systems. The Sokal score was developed in the 1980s for such a purpose, but has still proven to be relevant in the TKI era. Recently, the EUTOS Long-Term Survival (ELTS) score has been accepted as the preferred scoring system as it was developed in a TKI-treated population. Like the Sokal score, three groups are identified – patients at low, intermediate and high risk of disease progression. The variables measured at diagnosis to calculate the Sokal score and the ELTS score are the same; age, spleen size (measured in cm below the costal margin in the midclavicular line), platelet count and peripheral blood blast percentage. However, ELTS gives different weighting to each variable and has proven to be better at discriminating between different risk cohorts (Figure 33.6).

Imatinib

Imatinib was the first TKI to be licensed for use in CML, and for this reason, commonly referred to as a first-generation TKI. It competitively inhibits tyrosine kinases including c-kit, ABL1, ARG (*ABL2*), c-FMS and PDGFRB. It binds to inactivated forms of *BCR::ABL1* by occupying the ATP-binding pocket and



Number of patients still at risk (n) at different years of observation

Year	*0*	*3*	*6*	*9*
Low risk, n	1074	600	203	57
Intermediate risk, n	1177	598	175	50
High risk, n	698	341	82	19

Number of patients still at risk (n) at different years of observation

Year	*0*	*3*	*6*	*9*
Low risk, n	1688	935	302	86
Intermediate risk, n	853	427	115	34
High risk, n	408	177	43	6

Figure 33.6 Clinical risk scores in chronic phase CML predict cumulative incidence probabilities of death due to CML. Both the Sokal (Figure 33.6 (a) and the ELTS (Figure 33.6 (b) scores combine age, spleen size (measured in cm below the costal margin in the midclavicular line), platelet count and peripheral blood blast percentage into a single risk score to stratify patients into low, intermediate versus high risk of disease progression. The ELTS score has improved ability to discriminate patients at high risk of disease progression in the TKI era (Source: Adapted from Pfirrmann M, Clark RE, Prejzner W *et al.* (2020) The EUTOS long-term survival (ELTS) score is superior to the Sokal score for predicting survival in chronic myeloid leukaemia. *Leukemia* **34**: 2138–49. Reproduced with permission from Nature Publishing under a creative commons license.)

prevents conformational changes (such as repositioning of the 'activation loop') required for *BCR::ABL1* activation.

The Phase III IRIS trial enrolled treatment-naïve CML-CP patients and randomised them to receive either imatinib 400 mg daily, versus interferon-α and low-dose cytarabine. The superiority of the imatinib arm was demonstrated unequivocally, leading to crossover of 89.2% of the control arm patients by 2 years of follow-up. After 10 years, 83% of patients randomised to the imatinib arm were still alive. However, it is important to note that only around 50% of those patients were still receiving imatinib. Many patients had switched to another TKI because of intolerance or treatment failure.

As the IRIS study was the first to demonstrate the effectiveness of TKIs in CML, it was also in a position to examine the prognostic significance of treatment milestones. For instance, the 5-year analysis showed progression-free survival (defined in IRIS as progression to AP/BC, loss of a CHR or major cytogenetic response) to be 97% for patients who achieved CCyR by 12 months, versus 93% for patients who achieved only PCyR and 81% for patients who achieved less than PCyR. Of the patients who achieved CCyR, those who achieved the even deeper response of MMR had a PFS of 100%. Results from other TKI studies subsequently confirmed the prognostic utility of these molecular milestone responses, independent of the TKI chosen for treatment. (Figure 33.7).

Even though imatinib treatment has proved highly successful for many, ~40–50% of patients who start imatinib therapy will discontinue – about half for intolerance and half for resistance. The most common resistance mechanism identified is kinase domain mutations (see below). Clonal evolution, such as acquisition of an extra copy of the Ph^+ chromosome or development of isochromosome 17q may also be involved. Non-adherence, whether intentional, due to toxicity or financial pressures, or unintentional, is also a significant contributor to therapeutic failure. Some mechanisms of treatment failure are illustrated in Figure 33.8.

The most common starting dose of imatinib is 400 mg daily. A dose of 600 mg daily may lead to higher molecular responses and is well tolerated in many patients. However, randomised studies comparing imatinib 400 mg daily to 800 mg daily have not demonstrated a clear benefit to justify the uniform use of higher doses. The more common toxicities of imatinib tend to be gastrointestinal disturbances (nausea, vomiting, anorexia, diarrhoea and dyspepsia) and oedema (periorbital, facial and pedal). Judicious use of

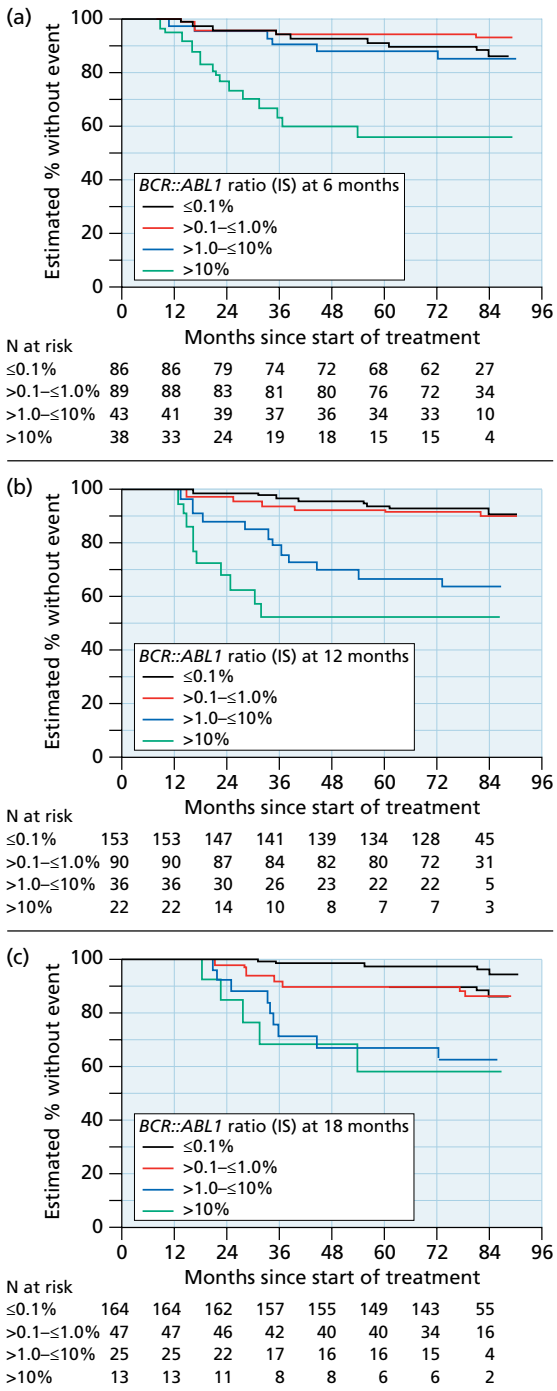


Figure 33.7 Event-free survival (EFS) from the IRIS cohort. The majority of IRIS patients enjoy excellent EFS. However, patients *BCR::ABL1* RQ-PCR of >10%, >1% and >0.1% at (a) 6, (b) 12 and (c) 18 months after starting therapy had inferior EFS. Events in this analysis included (i) loss of complete haematologic response, (ii) loss of major cytogenetic response, (iii) progression to AP/BC or (iv) death due to any cause (Source: Hughes *et al.* (2010) *Blood* **116**: 3758–65. Reproduced with permission of American Society of Haematology.)

diuretics, prokinetic agents and taking imatinib with a full meal may ameliorate these symptoms. Haematological toxicities (anaemia, neutropenia and thrombocytopenia) are commonly associated with TKIs and tend to be most severe at treatment initiation. Cardiac toxicity, an initial concern, appears minimal with long-term clinical follow-up. Unlike the second and third-generation, TKIs there is no evidence that the risk of cardiovascular events is increased by imatinib.

Nilotinib

Nilotinib is a second-generation TKI that is approved for frontline therapy as well as in patients with imatinib-resistance or -intolerance. In addition to *BCR::ABL1*, it also inhibits PDGFR and c-kit. It is active against a range of imatinib-resistant kinase domain mutations, except for T315I, F359V/C, E255K/V and Y253H/F (see later section for full details of mutation analysis). It is administered as a twice-daily dose on an empty stomach. Treatment is usually well tolerated apart from cytopenias at treatment initiation. Common associated biochemical abnormalities include elevations in serum liver enzymes, lipase and amylase and hyperbilirubinaemia (thought to be secondary to decreased hepatic bilirubin excretion into bile and is generally reversible). Pancreatitis is an uncommon, but notable idiosyncratic toxicity. The incidence of cross intolerance with imatinib is low with regards to non-haematological side-effects. Although all TKIs can prolong QTc interval, the effect of nilotinib is perhaps the best documented, especially where increased serum drug concentration results from enhanced absorption, when nilotinib is taken with a fatty meal.

The data surrounding frontline use of nilotinib came from the Phase III ENESTnd study, which showed CML-CP patients (at either 300 mg BID or 400 mg BID) have decreased risk of disease transformation to AP/BP, reduced risk of kinase domain mutation acquisition, and higher achievement of MMR/MR[4.5], compared to imatinib-treated patients. Nilotinib-treated patients were also more likely to achieve early molecular response (EMR; *BCR::ABL1* <10% at 3 months), a surrogate for progression-free survival (PFS) (Figure 33.9). However, overall survival differences were marginal between imatinib and nilotinib. While efficacy was similar in the nilotinib 300 mg BID and 400 mg BID arms, toxicity is more common at higher doses. Consequently, first-line patients are usually given 300 mg BID. Nilotinib-treated patients have higher incidence of hyperglycaemia and dyslipidaemia, and accelerated development of arterial occlusive disease is a major concern. This manifests as ischaemic heart disease, strokes and peripheral arterial occlusive disease and was shown to be significantly more common in both nilotinib arms compared to the imatinib arm in the ENESTnd study. A recent update showed the higher annual rate of vascular events associated with nilotinib treatment persists over the full 10 years of the study, with vascular events reported

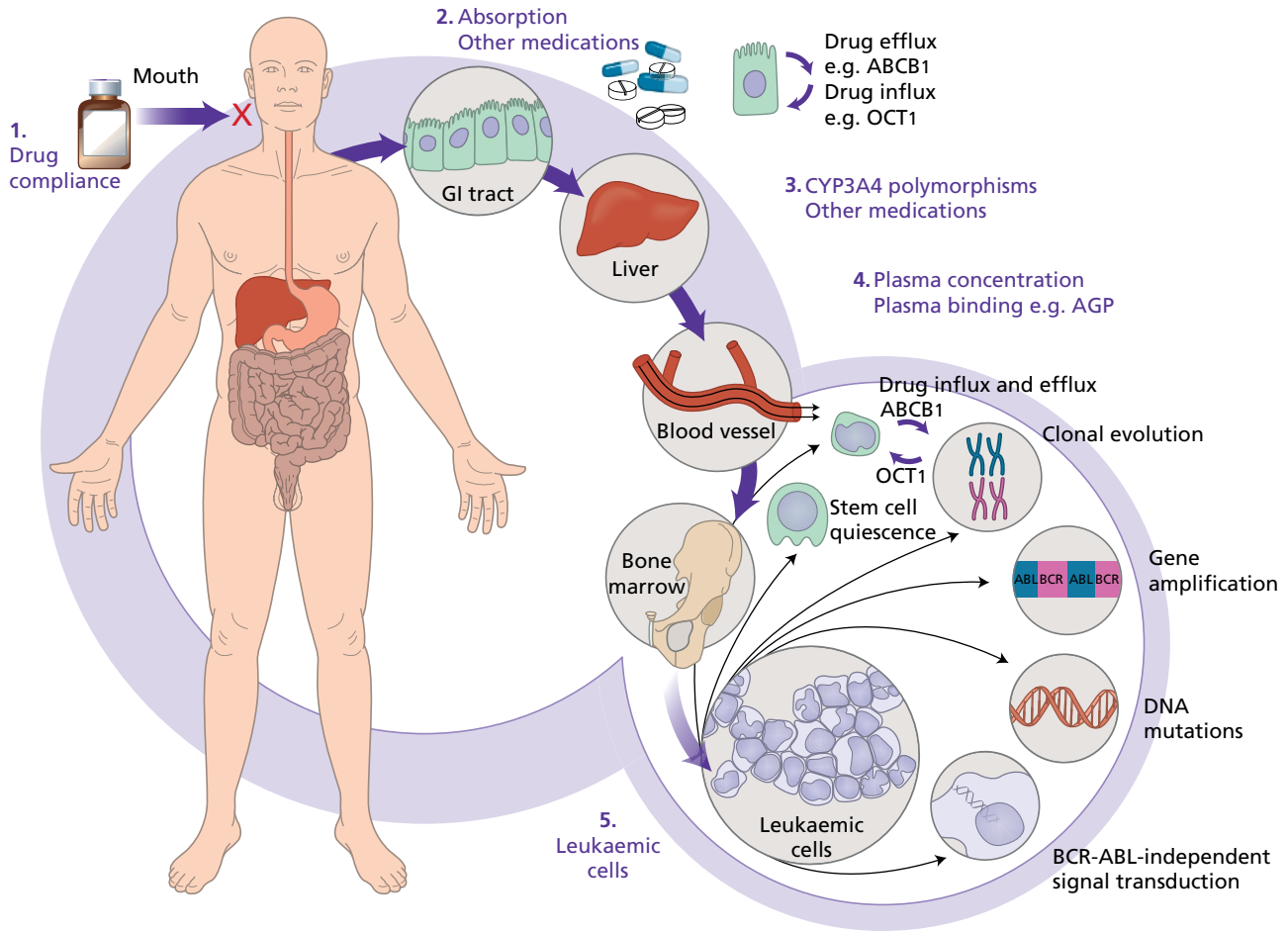


Figure 33.8 Mechanisms are implicated in treatment resistance. These factors include host factors drug compliance; absorption and metabolism. Concomitant medications that may interfere with pharmacokinetics. Factors, such as drug efflux, *BCR::ABL1* amplification and kinase domain mutations, as well as *BCR::ABL1* independent signalling contribute to leukaemia specific mechanisms of resistance. AGP, α-1 acid glycoprotein (Source: Apperley (2007) *Lancet Oncol.* **8**(11): 1018–29. Reproduced with permission of Elsevier.)

in 16.5, 23.5 and 3.6% of patients, respectively, in the nilotinib 300 mg BID, nilotinib 400 mg BID and imatinib arms. The Framingham risk score, which sums up predisposing risk factors for cardiovascular events such as smoking, hypertension, dyslipidaemia, age and being male, was predictive of cardiovascular events on nilotinib treatment. Proactively addressing modifiable risk factors with lifestyle modifications, and pharmacotherapy when appropriate, are all necessary to minimise morbidity and mortality from cardiovascular complications for nilotinib-treated patients.

Dasatinib

Dasatinib is a second-generation TKI and a dual Src/ABL inhibitor which also inhibits a number of other tyrosine kinases. It is approved for frontline treatment of CML-CP, as well as cases resistant or intolerant to at least one prior TKI in all phases, and for Ph^+ ALL. The initial registration studies used a twice-daily treatment regime based upon dasatinib having a short plasma half-life. However, dasatinib should be given as a single daily dose (100 mg for CP and 140 mg for advanced phases), taken with or without food due to reduced incidence of toxicity without a reduction in efficacy compared with the twice-daily regimen. In the Phase III DASISION study, dasatinib-treated patients had superior achievement of MMR and $MR^{4.5}$ compared to imatinib treatment, though no significant difference in overall survival and progression-free survival was demonstrated. Interestingly, the number of patients who developed kinase domain mutations was similar in both arms, with a higher number of T315I cases in dasatinib-treated patients. In the second-line setting, dasatinib is active against a range of imatinib-resistant mutations, although it is inactive against *T315I/A*, *V299L* and *F317V/C* mutations.

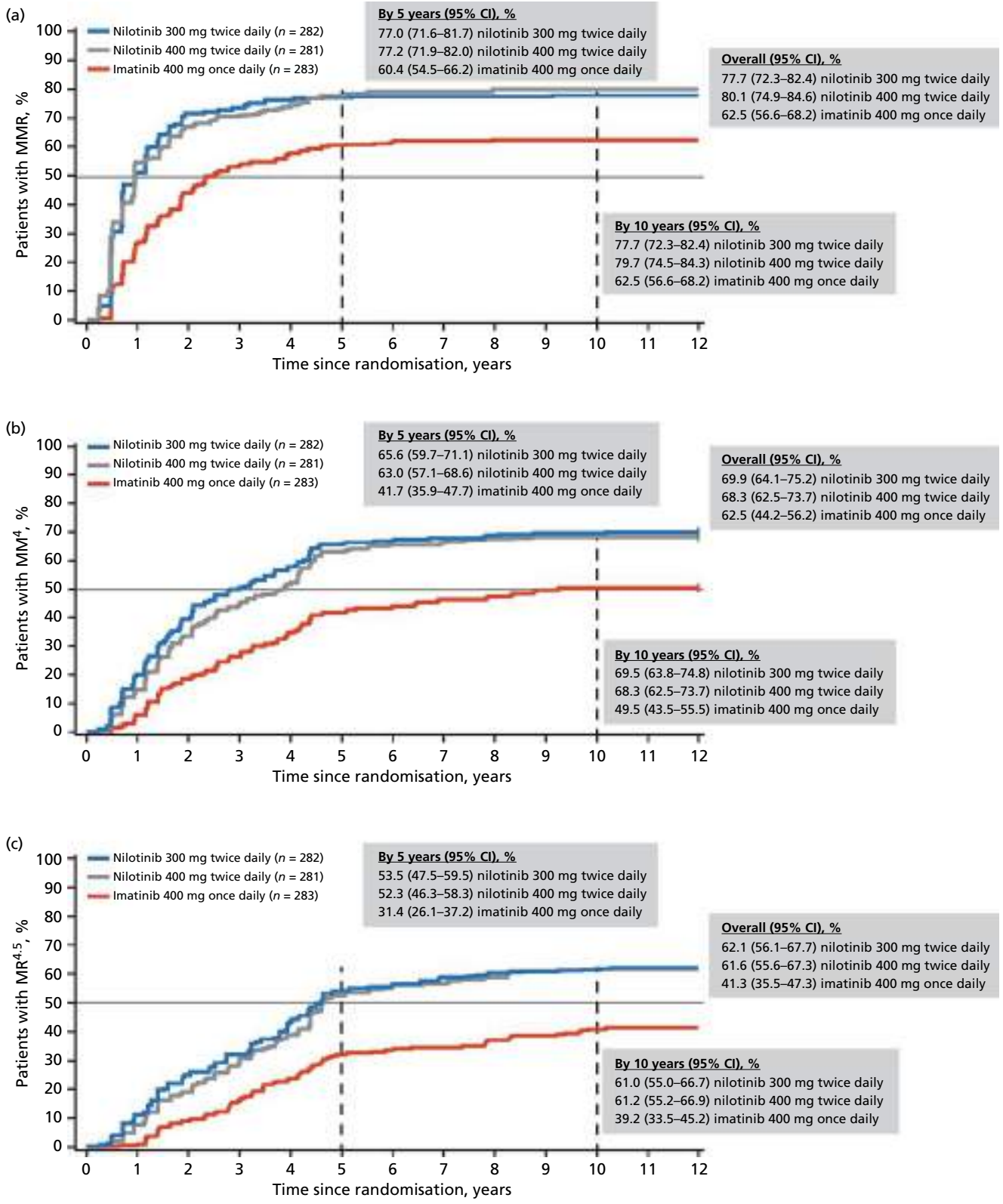


Figure 33.9 Results from the randomised ENESTnd study comparing the second generation TKI, nilotinib, to imatinib, in newly diagnosed CML-CP patients. Achievement of MMR, MR4 and MR4.5 in panels (a), (b) and (c) respectively. Subjects were randomised to three arms – nilotinib 300 mg BID, nilotinib 400 mg BID versus imatinib 400 mg OD. Patients who received nilotinib had superior probability of achieving MMR and $MR^{4.5}$. Similarly, data from the Phase III DASISION study showed that patients assigned to the second-generation TKI dasatinib had better molecular responses compared to patients receiving imatinib (Source: Kantarjian *et al.* (2021) *Leukemia*. **35**: 440–53. Reproduced with permission from Nature Publishing under a Creative Commons license for Open Access.)

Idiosyncratically, dasatinib causes pleural effusions in some patients. The most important risk factor appears to be age, with patients over 70 years of age having an incidence of over 50%. Onset of pleural effusions are unpredictable and may first present months to years after treatment commencement, though the risk is cumulative with increasing duration of treatment. The pleural fluid is usually an exudate, often associated with lymphocytosis which may be oligoclonal, suggesting a degree of immune dysregulation in its aetiology. Usual treatment of symptomatic, dasatinib-induced and pleural effusions include a brief treatment interruption and judicious use of thoracentesis as necessary. A short course of prednisolone can be used as immunomodulation, but no firm evidence exists to demonstrate the effectiveness of this approach. Switching to an alternate TKI may be necessary in some patients, if pleural effusions do not resolve with these simple measures. Occasionally, the pleural effusion may persist after switching to another TKI. Pulmonary hypertension (PAH) appears to be a rare complication of dasatinib therapy, though given the difficulty in diagnosing PAH and its inconsistent symptoms, the true incidence is difficult to ascertain. This is usually, but not always, reversible upon dasatinib cessation, although pulmonary arterial pressures may not return to normal. Other idiosyncratic side effects associated with dasatinib treatment include pericardial effusions, lymph node follicular hyperplasia and peripheral blood lymphocytosis. It also inhibits platelet activation *in vitro*, though it is unclear whether this is clinically relevant in the general case.

The safety and efficacy of lower doses of dasatinib are currently being assessed. In one single institution study, dasatinib given at 50 mg daily was associated with similar efficacy and substantially lower risk of toxicity, including pleural effusions, as compared to historical controls using 100 mg daily. These data are awaiting further confirmation. Data also exist to suggest a correlation between dasatinib trough plasma levels, and the risk of pleural effusions. In the French-Canadian OPTIM study, patients with dasatinib trough levels ≥3nM were randomised to having serial dose reductions aiming for a plasma trough <3nM, versus no intervention. The cumulative incidence of pleural effusion at 36 months was 45% in patients with a dasatinib trough level ≥3nM without dose reduction, versus 11% in those who had a dose reduction. Molecular response was similar regardless of starting plasma trough dasatinib level. The lack of availability of laboratories that offer a dasatinib trough assay may hamper widespread adoption of this approach.

Bosutinib

Bosutinib is a second-generation TKI approved for the frontline treatment of CML patients as well as in cases of intolerance or resistance to other TKI, for all disease phases. Its efficacy in the frontline setting was demonstrated in the Phase II BFORE study where 536 newly diagnosed CML-CP patients were randomised to bosutinib versus imatinib in a 1:1 ratio, both at 400 mg daily. The primary endpoint, MMR at 12 months was significantly higher in bosutinib-treated patients (47.2%) as compared with imatinib-treated patients (36.9%). Disease progression rate to CML-AP/CML-BP was 1.6 versus 2.5%, respectively, in patients treated with bosutinib and imatinib.

Bosutinib is also approved in the second-line setting, though clinical evidence suggests inferior responses for patients with the V299L, L248V and F359C (and perhaps G250E) mutations. The T315I mutant clone is totally resistant to bosutinib. The Phase 4 BYOND study recently reported a 12-month MMR rate of 70.5% in 163 CML patients resistant or intolerant to at least one prior TKI treatment. Patients received 500 mg daily of bosutinib on this study, and those with the *T315I* and *V299L* mutations were excluded.

Unlike the other second-generation TKIs, Bosutinib has not been associated with an increased risk of vaso-occlusive events. The main toxicities observed are gastrointestinal, particularly diarrhoea and elevated liver enzymes. (Grade III diarrhoea was observed in 7.8% of patients in the BFORE study). These toxicities are quite common in the first and second year of therapy, leading to a discontinuation rate due to adverse events of around 20–25%. These toxicities can often be managed successfully with dose reductions over the first few months of therapy.

Ponatinib

Ponatinib, a third-generation TKI, and was the first drug approved for use against the *T315I* mutation – a mutation that confer resistance to all other available TKIs. Ponatinib is often effective in patients with resistance or intolerance to at least one previous line of treatment in all phases of CML disease, as well as Ph^+ ALL, especially in patients with T315I as the only known mechanism of resistance. Cases with compound mutations (where more than one kinase domain point mutation is present in the same *BCR::ABL1* transcript), especially those involving the *E255K/V* mutation, are often resistant to ponatinib. Rash, elevated lipase, pancreatitis and headaches seem to be common non-haematological toxicities of the drug. Of significant concern, 27% of patients who participated in early clinical studies reported having had either arterial or venous thrombotic events. The EPIC study that randomised newly diagnosed CML-CP patients to imatinib versus ponatinib was prematurely terminated due to these concerns. The recently reported OPTIC study suggested a correlation between dose and risk of vascular events. This dose-finding study in CML-CP patients either resistant or intolerant to other TKIs randomised to a ponatinib starting dose of 45, 30 versus 15 mg. Patients assigned to the 45 and 30 mg arms were dose reduced to 15 mg once *BCR::ABL1* falls below 1%. This was achieved in 44.1, 29.0 and 23.1% of patients in these 3 arms respectively. The exposure-adjusted rates of arterial occlusive events were 5.6, 3.6 and 2.1% respectively, with a median follow-up of 32 months. The molecular response was superior in the cohort who were initially started on ponatinib at a dose of 45 mg/day. All 3 arms had significantly

lower rates of vascular events compared to earlier studies, such as the PACE trial, where higher doses than 15 mg/day were often maintained long-term. As with nilotinib, proactive identification and management of cardiac and vascular disease risk factors are strongly advised for patients receiving ponatinib.

Asciminib

Asciminib (earlier designated as ABL001) is a TKI with a different mode of action compared to all of the currently available TKIs. Rather than competitively blocking ATP binding, asciminib binds to a myristoyl pocket on Abl which leads to inactivation of kinase activity. Its sponsor, Novartis, has coined the term STAMP (Specifically Targeting the ABL Myristoyl Pocket) to differentiate it from TKIs that bind to the ATP-binding pocket. Asciminib selectively binds to *ABL1*, *ARG* (*ABL2*) and *BCR::ABL1*, with significantly less off-target effects that are a major issue for all of the ATP competitive TKIs. In the phase I study, asciminib demonstrated a good safety profile and proved to be efficacious in many patients who were intolerant and/or resistant to several TKIs. In the Phase III randomised ASCEMBL study, it was shown to be superior to bosutinib in patients who had failed at least 2 other TKIs and is now approved for use in the third-line setting in many jurisdictions. Resistance to asciminib has been observed, in some case this is due to mutations within the myristoyl pocket, such as V468F, P465S, I502L and A337V. The kinase domain mutations that lead to resistance to ATP-competitive TKIs are often sensitive to asciminib, though T315I and F359V are less sensitive to asciminib than the native form of *BCR::ABL1*. An expanded arm of the Phase I study used a higher dose of asciminib (200 mg twice daily to specifically target T315I, as compared to the standard dose of 40 mg twice daily) showed good efficacy and comparable toxicity to the standard dose. This is also an approved indication in many jurisdictions. The most common toxicity observed with asciminib therapy has been thrombocytopenia and an abnormally elevated lipase which is sometimes associated with clinical pancreatitis. Asciminib represents a promising new therapeutic option for many CML patients and the ASC4FIRST study has recently demonstrated superior efficacy for achievement of MMR versus investigator selected TKI (68% versus 49% at week 48) in the first line setting.

Choice of TKI upfront

Currently, imatinib, nilotinib (300 mg BID), dasatinib and bosutinib (400 mg/day) are available in most countries for up-front treatment in CML-CP. All four are reasonable options. Although the second-generation TKIs lead to improved molecular responses, all four lead to similar long-term overall survival. The choice of therapy for each individual patient would rest mainly on a patient's comorbidities and their risk of disease transformation, as well as the priority placed on achievement of deep molecular responses and opportunities for a subsequent attempt at treatment cessation.

The second generation TKIs may be reasonable choices in patients with high ELTS risk. The daily dosing of dasatinib may be more convenient in younger patients at higher risk of non-compliance, and there is emerging data that a reduced dose of 50 mg daily may be well tolerated even in the elderly and shows equivalent efficacy to the standard dose of 100 mg daily. Dasatinib should generally be avoided in patients with significant coexisting cardiopulmonary disease who risk decompensation with pleural effusion and pulmonary hypertension. Bosutinib is also taken once a day, though its side effect of diarrhoea may be troubling for patients. Nilotinib appears to have a similar efficacy with regards to achievement of deep molecular responses compared to dasatinib and may be ideal in patients for whom the opportunity to cease treatment is a high priority (e.g. women planning to start a family). However, its twice-daily dosing away from food is challenging for many patients and may exacerbate nonadherence. Nilotinib should be avoided in patients with previous vascular events and poorly controlled cardiovascular risk factors, especially at the higher dose of 400 mg BID.

Imatinib, in contrast, appears to have a favourable long-term toxicity profile. While side-effects are common and cause significant discomfort, most patients learn to manage these symptoms. In older patients with pre-existing vascular or respiratory problems, imatinib would often be a better choice. However, patients who receive imatinib upfront at a dose of 400 mg daily are at higher risk of transformation to AP/BP, in comparison to those who receive up-front nilotinib or dasatinib.

Incorporation of both imatinib and a second-generation TKI into a combined treatment strategy may be a reasonable compromise to enhance safety and retain efficacy. For instance, the Australasian TIDEL-II study started newly diagnosed CML-CP patients on imatinib 600 mg daily. Patients were assessed against time-dependent molecular targets (*BCR::ABL1* $\leq$10%, $\leq$1% and $\leq$0.1% at 3, 6 and 12 months, respectively). Patients who failed these targets switched to nilotinib with or without a trial of imatinib 800 mg/day, as did patients with imatinib intolerance or loss of response. The 24-month MMR, MR4.5 and the 3-year overall and progression-free survival from TIDEL-II were similar to that achieved by patients who received a second-generation TKI up front. However, this strategy allowed many patients to stay on imatinib. Over the first 2 years of study treatment, ~50% of patients remained on imatinib and ~30% of patients switched to nilotinib, while ~20% had discontinued study treatment.

In the paediatric setting, the bulk of evidence and experience concerns the use of imatinib, where the efficacy and side-effect profile are very similar to that seen in adults. Imatinib, as with other TKIs, is dosed according to body surface area in children (260–340 mg/m^2/day). The second-generation TKIs dasatinib and nilotinib are also approved for use for paediatric CML in many jurisdictions, dosed at 60 mg/m^2/day and 230 mg/m^2 twice a day for the 2 agents respectively. It is important to monitor for growth, pubertal and physical development of children treated with TKIs, as various degrees of growth deceleration have been

reported. Imatinib is known to cause altered vitamin D metabolism, hypocalcaemia, hypophosphataemia and decreased bone remodelling, all factors that may lead to stunted growth. Growth and maturation may also be affected through interference with sex hormone metabolism, and endocrinology assessment may be necessary in children with delayed maturation. Cardiovascular toxicities have generally not been as much a concern here as in adult CML patients although longer-term follow-up is limited.

Treatment goals

For CML-CP patients treated with TKIs, disease-specific survival is excellent and overall survival is similar to age-matched controls. However, despite the introduction of increasingly potent TKIs, survival for patients with CML-BP has not improved dramatically, and prevention of disease transformation is therefore of paramount importance. There is a reliable correlation between time-dependent reductions in tumour burden (measured by RQ-PCR or cytogenetics) and survival. Milestone responses, such as achievement of *BCR::ABL1* levels below 10% (IS) at 3 months, 1% (IS) at 6 months and below 0.1% (IS) at 12 months, differentiate patients at high risk of disease transformation from patients with optimal treatment response. MMR at 12 months in particular has been adopted as surrogate end points for many clinical trials.

Consensus statements concerning optimal time-dependent treatment targets are available. The two most widely used are published by the US-based NCCN (National Comprehensive Cancer Network) and the ELN (European Leukaemia Net), with subtle differences between the two. The ELN stratify responses at 3, 6 and 12 months after therapy commencement into three categories – Optimal, Warning and Failure. Optimal response is associated with favourable long-term survival. Conversely, patients who fail any of the time-dependent targets are at high risk of disease progression should they continue with the same treatment. Patients who have received a 'warning' are in the intermediate category and these patients should be closely monitored and counselled to adhere to therapy. Similarly, the NCCN uses a traffic light system where patients at highest risk of disease progression belong to the red category, versus patients with possible/emerging TKI resistance in the yellow category, versus patients who have achieved their treatment targets in green.

The relevant milestones are summarised in Table 33.2. Treatment recommendations are based on consensus expert opinions, as good-quality evidence demonstrating the benefit of interventions is not currently available. Interventions based on early molecular responses are a particularly contentious issue. Patients who record a *BCR::ABL1* IS >10%, 3 months after starting therapy have inferior overall survival, a high risk of disease transformation (between 8 and 12%), and inferior achievement of MMR/MR$^{4.5}$ (Figure 33.10). ELN recommendations advise that a *BCR::ABL1* level >10% (IS) at 3 months, if confirmed on a second test, is consistent with treatment failure. Interestingly, data from the Adelaide cohort of imatinib-treated patients suggest that patients with *BCR::ABL1* >10% at 3 months may be further risk stratified, based on an individual patient's *BCR::ABL1* halving time. Patients with a halving time of >76 days were at the highest risk of treatment failure. The German CML IV study group came to the similar conclusion that the early velocity of an individual patient's *BCR::ABL1* drop may add additional prognostic information to RQ-PCR measurements at single time points.

A check for medication adherence and drug interactions are advised for any patient who have not achieved their treatment targets (NCCN Green/ ELN Optimal). Mutational analysis is useful and may guide TKI selection for subsequent line therapy. Continuing imatinib at a dose of 400 mg daily is generally not recommended, although an increase in the imatinib dose to 600 mg daily is an effective strategy for some patients.

Table 33.2 Consensus time-dependent molecular targets from the US National Comprehensive Cancer Network, 2021 edition; and the European Leukaemia Net, 2020 edition. Refer to text for recommended management options for each category.

Time point	*BCR::ABL1* QPCR	NCCN	ELN
3 months	>10%	Possible TKI resistance	Failure, if confirmed within 1–3 months
	≤10%	TKI-sensitive disease	Optimal
6 months	>10%	TKI-resistant disease	Failure
	>1% but ≤10%	TKI-sensitive disease	Warning
	≤1%	TKI-sensitive disease	Optimal
12 months	>10%	TKI-resistant disease	Failure
	>1% but ≤10%	Possible TKI resistance	Failure
	>0.1% but ≤1%	TKI-sensitive disease. Response optimal for survival but not TFR	Warning
	≤0.1%	TKI-sensitive disease	Optimal

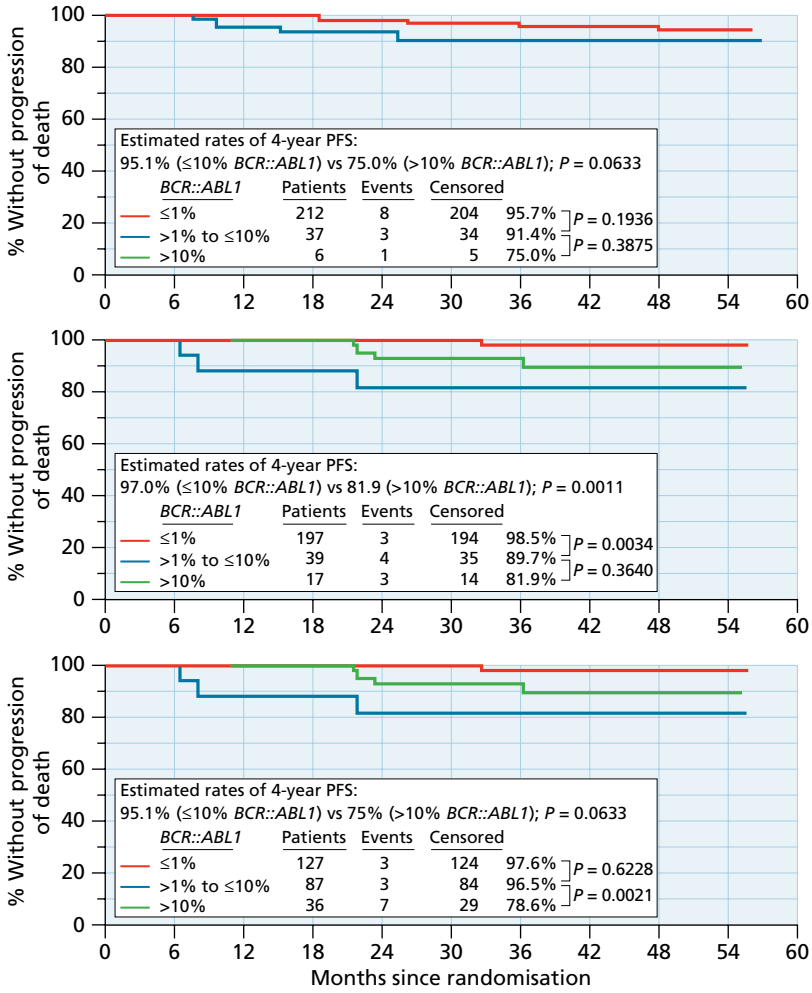


Figure 33.10 Patients failing to achieve an early molecular response experience inferior survival regardless of frontline therapy received. These results from the ENESTnd study showed progression-free survival to be 75.0, 81.9 and 78.6% in the nilotinib 300 mg BID, nilotinib 400 mg BID and imatinib 400 mg OD arms, respectively, in patients with *BCR::ABL1* >10% at 6 months. (Top panel – nilotinib 300 mg BID, middle panel – nilotinib 400 mg BID, bottom panel – imatinib 400 mg OD). In comparison, patients with *BCR::ABL1* ≤1% 6 months after starting therapy enjoyed 4-year progression-free survival of 95.7–98.5%. The same conclusion can be drawn from the landmark analyses from the DASISION study comparing imatinib to dasatinib (Source: Hughes *et al.* (2014) *Blood* **27**; **123**(9): 1353–60. Reproduced with permission of American Society of Haematology.)

For patients in the NCCN yellow and ELN warning category, switching to an alternative TKI is not mandatory in the absence of a resistant kinase domain mutation, especially if there is a clear downward trend in the *BCR::ABL1* QPCR. Selection of an alternative TKI depends on the priority for early treatment discontinuation as well as the patient's comorbidity, matched for tolerance of likely toxicities. For patients in the NCCN Red/ELN Failure category, switching to an alternative TKI is recommended. Workup for allogeneic stem cell transplant may be warranted in patients otherwise fit for the procedure, and where no benefit is demonstrated with alternative TKI therapy.

Duration of treatment

With the success of TKI therapy, an increasing number of patients achieve deep and durable molecular responses. Although it was originally assumed that patients would need to continue TKI therapy indefinitely to prevent late disease relapses, a number of patients have since participated in treatment cessation studies and have achieved treatment-free remissions (TFR). TFR – defined as the maintenance of MMR in the absence of further treatment in patients who have previously achieved sustained DMR with TKIs – is now the goal of CML treatment for many patients. Eliminating the need for further TKI therapy not only minimises the impact of existing side-effects and removes the potential for long-term complications, but also decreases the overall financial burden associated with drug costs.

Two of the earliest treatment cessation studies were the French STIM study and the Australian TWISTER study (Figure 33.11), where imatinib-treated CML-CP patients who had undetectable *BCR::ABL1* (with assays of >5 log sensitivity) for an uninterrupted period of 2 years stopped imatinib and were monitored monthly with RQ-PCR. Rapid molecular relapse (i.e. RQ-PCR moving from undetectable to positive) was noted in 60% of patients, mostly occurring within the first 6 months after stopping. All relapses were sensitive to imatinib re-challenge, with most patients re-establishing deep molecular response, and no disease transformation was reported.

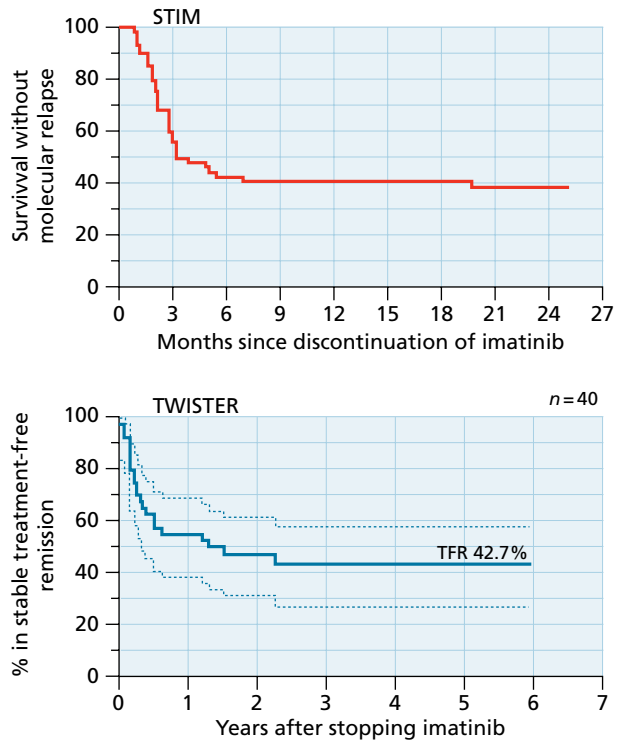


Figure 33.11 For patients with deep molecular responses, a trial of TKI cessation may lead to durable treatment-free remissions (TFR). The STIM (top panel) and TWISTER (bottom panel) studies both showed the rate of TFR in patients who maintained MR^5 for 2 continuous years prior to trial of therapy cessation to be ~40% (Source: (Top panel) Hughes *et al.* (2014) *Blood* **123**(9): 1353–60. (Bottom panel) Ross *et al.* (2013) *Blood* **122**(4): 515–22. Reproduced with permission of American Society of Haematology.)

The remaining 40% have remained in a deep molecular remission off treatment, many for over 10 years. There have been, as yet, no late relapses reported in this group, raising the possibility that TFRs may truly be durable in the long term. These data are corroborated by a number of subsequent studies involving patients treated with imatinib as well as second-generation TKIs. Interestingly, the proportion of patients who remain in TFR falls between 40 and 60% long-term, regardless of the potency of TKI used initially for treatment. Indeed, the biological determinants of successful treatment cessation remain elusive, but appear to correlate with total TKI treatment duration as well as duration of a deep molecular response. This may relate to senescence of Ph+ clones over long periods of TKI treatment. Additionally, patients with a more rapid reduction in *BCR::ABL1* transcripts during their first 3 months of TKI treatment, as well as patients with the e14a2 transcript, appear to have a higher TFR success rate. Not all patients who experience early molecular relapse require retreatment. In about a third of these patients, RQ-PCR becomes detectable, but remains below 0.1%, even without treatment.

Given that a number of studies with differing TFR eligibility criteria all led to similar TFR success rates, there is still uncertainty regarding the minimum criteria a patient has to achieve before being offered a TFR attempt (Table 33.3). For instance, the ENESTfreedom study allowed TFR attempts with a minimum of 3 years of nilotinib treatment, including a 12-month period of MR4.0 response and MR4.5 to be achieved immediately prior to treatment cessation. This information has since been included as part of nilotinib's approved product information in US and EU. However, the consensus criteria for TFR is more conservative, with the example from the ELN 2020 guidelines representing a reasonable approach (Table 33.4). CML-CP patients with no prior history of treatment resistance, who have had TKI therapy totalling 5 year or more, and have been in DMR for 2–3 years, are eligible for TFR in this guideline. An alternative approach to toxicity minimisation may be dose reduction. Recently, the UK DESTINY study showed that patients with stable DMR may safely reduce their TKI dose by 50% for 12 months, before a planned discontinuation. Patients in stable MR^4 in this study showed a particularly high molecular recurrence-free survival rate (RFS) of 76% at 24 months. However, whether an initial dose de-escalation strategy improves rates of successful long-term TFR remains uncertain in the absence of randomised trial comparison with conventional TFR approaches.

Further studies are currently underway to examine whether adjunctive therapy such as pegylated interferon will be of benefit just prior to or in the initial period of therapy cessation. The optimal criteria for a second TFR attempt in patients who previously had one failed attempt at TFR also remains to be determined. Of note, current recommendations for TFR should not be extrapolated to patients with current or previous advanced-phase disease.

Treatment resistance

Treatment resistance may be encountered in ~10–20% of patients receiving TKI in the frontline setting (an additional ~10–20% of patients fail therapy because of varying degrees of toxicity, and some are better classified as failing therapy because of intolerance, though the two frequently overlap). Kinase domain mutations are identified in 30–50% of cases and are the most frequently identified mechanism of treatment resistance. These point mutations result in amino acid changes, which decrease binding affinity of TKIs (Figure 33.12). The consequence is restoration of constitutive kinase activity. Imatinib appears to be susceptible to the widest range of mutations – over 50 have been documented. The second-generation TKIs are active against most imatinib-resistant mutations, though the T315I mutation confers resistance to all TKIs except ponatinib and asciminib. This threonine residue at position 315 in the ATP binding pocket is critical to the binding of most TKIs and is substituted for isoleucine in ~10–15% of mutations. Other commonly/encountered mutations of note include the *V299L*

Table 33.3 Summary of representative treatment-free remission studies in the context of second-generation TKIs. Regardless of the TKI used in the initial treatment, once a patient achieves deep molecular response and maintains this for 12 or more months, the probability of sustaining molecular response upon treatment cessation is ~45–60%, similar to results reported by imatinib-stopping studies.

Study	*n**	TKI and line of treatment	Median duration of treatment with TKI (total and second-generation	Qualifying DMR	Median duration of DMR	Definition of molecular relapse	Percentage (%) of patients in TFR
First-line DADI[39]	58	Dasatinib, first	40 months	MR4.0 for ≥1 yr	23 months	Loss of MR4.0	55 at 12 months
DADI[40,41]	63	Dasatinib, second	82 months and 17 months	MR4.0 for ≥1 yr	NR	Loss of MR4.0	44 at 36 months
D-STOP[42]	54	Dasatinib, first and second	92 months and NR	MR4.0 for ≥2 yrs	51 months	Loss of MR4.0	63 at 12 months
DASFREE[43]	84	Dasatinib, first and second	69 months and NR†	MR4.5 for ≥2 yrs	28 months	Loss of MMR	46 at 24 months
STOP 2G-TKI[44]	30 (each TKI)	Dasatinib and nilotinib, first and second	76 months and 39 months	UMRD for ≥2 yrs	29 months	Loss of MMR	54 at 48 months
STAT2 (ref.[45])	78	Nilotinib, second	99 months and 25 months	MR4.5 for ≥2 yrs	51 months	Loss of MR4.5	63 at 36 months
ENESTop[46]	126	Nilotinib, second	88 months and 53 months	MR4.5 for ≥1 yrs	32 months	Loss of MR4.0	53 at 96 weeks
ENESTfreedom[47,48]	190	Nilotinib, first	44 months	MR4.5 for ≥1 yr	30 months	Loss of MMR	49 at 96 weeks

DMR, deep molecular response; MR4.0/4.5, deep molecular response with *BCR::ABL1* mRNA levels in peripheral blood of ≤0.01%/0.0032%; MMR, major molecular response; NR, not reported; TFR, treatment-free remission; TKI, tyrosine-kinase inhibitor; UMRD, undetectable minimal residual disease.

* *n* refers to the number of patients in the study of TKI discontinuation for whom outcomes were reported.

† The total duration since diagnosis is provided, which approximates TKI duration.

(*Source:* Ross DM and Hughes TP (2020) *Nat. Rev. Clin. Oncol.* **17**: 493–503. Reproduced with permission from Nature Publishing.)

Table 33.4 Consensus ELN 2020 criteria for treatment free remission (TFR) attempts.

Mandatory
- CML in first CP only (data are lacking outside this setting)
- Motivated patient with structured communication
- Access to high-quality quantitative PCR using the International Scale (IS) with rapid turn-around of PCR test results
- Patient's agreement to more frequent monitoring after stopping treatment. This means monthly for the first 6 months, every 2 months for months 6–12, and every 3 months thereafter.

Minimal (stop allowed)
- First-line therapy or second-line if intolerance was the only reason for changing TKI
- Typical e13a2 or e14a2 *BCR::ABL1* transcripts
- Duration of TKI therapy >5 years (>4 years for 2GTKI)
- Duration of DMR (MR4 or better) >2 years
- No prior treatment failure

Optimal (stop recommended for consideration)
- Duration of TKI therapy >5 years
- Duration of DMR > 3 years if MR4
- Duration of DMR > 2 years if MR4.5

(*Source:* Adopted from Hochhaus A, Baccarani M, Silver RT *et al.* (2020) European LeukemiaNet 2020 recommendations for treating chronic myeloid leukaemia. *Leukemia*. **34**: 966–984. https://doi.org/10.1038/s41375-020-0776-2. Used with permission under an open access Creative Commons License.)

and *F317V/C* mutations resistant to dasatinib, and the *Y253H*, *E255K/V* and *F359C/V* mutations resistant to nilotinib. Mutational analysis should be performed when a patient fails to reach their time-dependent targets or has loss of a previously achieved response. Most importantly, screening for kinase domain mutations should always be done in patients who are resistant to a TKI, prior to selecting the most suitable TKI for ongoing therapy. Sanger sequencing is the traditional diagnostic method for mutation analysis. It is relatively cheap and more widely available, but can only detect mutant clones if they are present in >10–15% of the total *BCR::ABL1*+ population, and only when the total *BCR::ABL1* is >0.1% IS. Matrix-assisted laser desorption/ionisation with time of flight (MALDI-TOF, MassArray) is a specialised technique with a sensitivity as low as 0.1%, but only for specific mutations. Next-generation sequencing can detect mutations with a sensitivity as low as 3% and has recently been endorsed by the ELN for kinase domain mutation analysis. Data are also accumulating to suggests that advanced phase CML may be associated with mutations in certain molecular driver genes e.g. *ASXL1*. Screening for these mutations in patients with high-risk disease via NGS may, in the future, also give warnings of impending disease progression.

Advanced phase diseases

In advanced phase CML, TKI monotherapy is much less effective compared to CML-CP. Although a number of patients can achieve complete haematological responses, these are rarely durable without follow-up combination chemotherapy and allogeneic HSCT. Fortunately, over 90% of CML patients are diagnosed in CP, and with effective TKI treatment, only ~5% of these patients transform to AP/BP.

Evidence regarding the use of TKIs in *de novo* CML-AP is scant. In the STI571 0109 study, imatinib 600 mg daily led to a CCyR of 20%. In this study, response rates of AP patients with both clonal evolution and increased blast counts were inferior to those with either feature alone. Extrapolating from data with frontline TKI use in CML-CP patients, the more potent second-generation TKIs should be superior to imatinib in CML-AP. In the MD Anderson Cancer Centre cohort of 51 patients, imatinib led to overall survival of 87% at 3 years, with 46% of patients achieving MMR by 18 months. In contrast, patients treated with second-generation TKIs had a 3-year overall survival of 95%, with an 18-month MMR rate of 56%. It is reasonable therefore to treat CML-AP patients with TKI monotherapy in the first-line setting.

Outcomes for CML-BP patients remain dismal, even with potent TKIs, and patients invariably develop resistance to TKI monotherapy. Patients with lymphoid BP are best treated with Ph^+ ALL induction chemotherapy regimens. TKIs are of benefit when used prior to or in combination with chemotherapy, though this may compound marrow toxicity and delay cell count recovery. Whether TKIs have effective CNS penetration is unclear and standard ALL CNS prophylaxis should be offered. Combination of TKI with blinatumomab is an emerging treatment approach, although experience in lymphoid BP remains limited. Myeloid BP is best treated with AML-type induction regimens, with combinations such as cytarabine and an anthracycline. As with lymphoid BP, TKIs in combination with chemotherapy are generally preferred. Treatment responses in CML-BP are rarely durable and transplant-eligible patients should proceed to allogeneic HSCT once a second chronic phase has been established.

In patients with significant comorbidities or those otherwise unfit for chemotherapy, TKI monotherapy would be a reasonable option. As with CML-AP, there is a paucity of data regarding the use of TKI monotherapy in *de novo* CML-BP. In one Phase II study, 154 treatment-naïve CML myeloid BP patients were treated with imatinib. This led to CHR lasting for ≥4 weeks in 8.8%, and CCyR in 8.1%.

For patients who progressed to AP/BP from CP while on treatment, the choice of the subsequent agent should be directed by results of the mutation analysis. About a third of these patients will achieve CCyR with a second-line agent. The T315I mutation is one of the most commonly found mutations in this context and will preclude successful use of all second-generation TKIs. The PACE study included patients with treatment resistance to

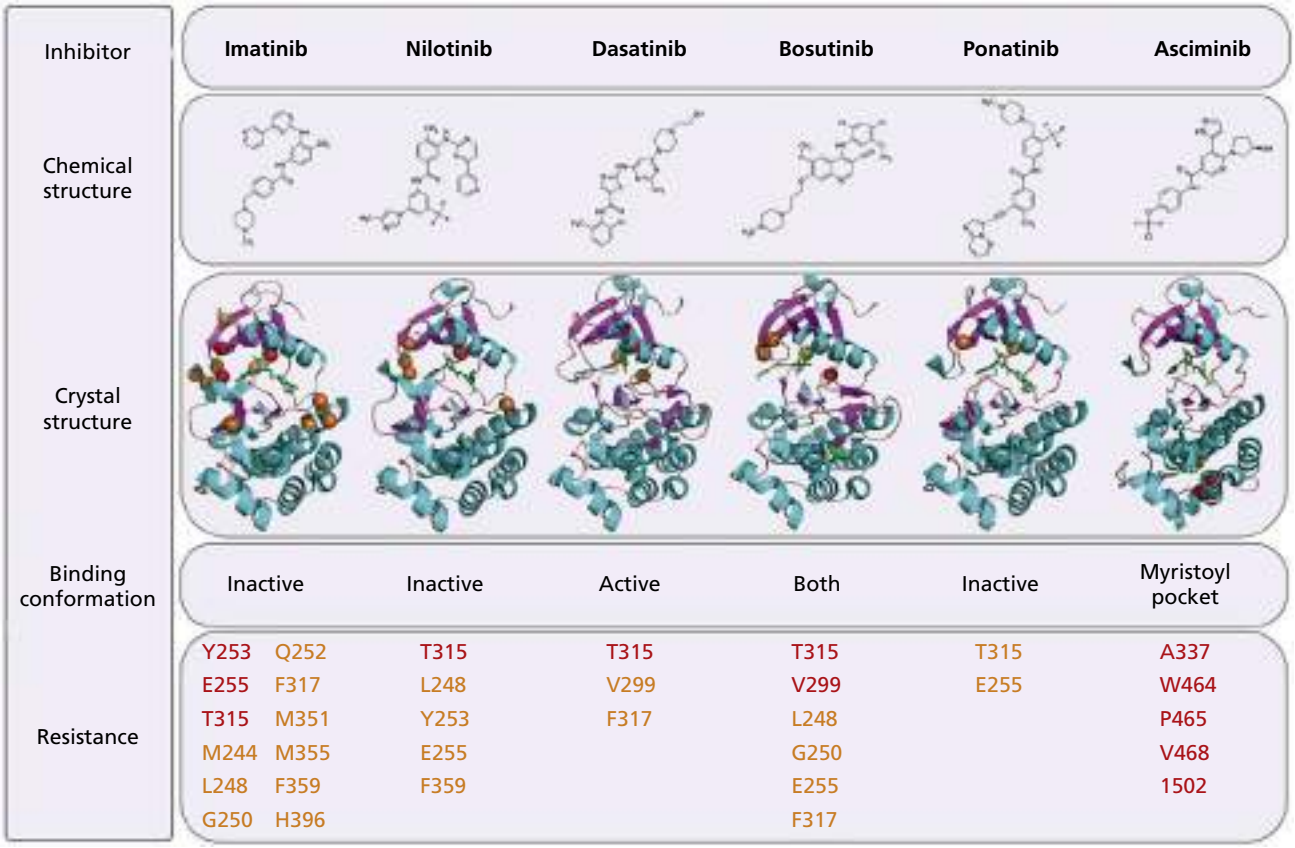


Figure 33.12 Chemical structures of *BCR::ABL* inhibitors currently in clinical use, and the published X-ray crystallographic structures of ABL1 as complexed with these drugs. Bottom panel lists mutations that confer resistance to these specific inhibitors. (High-level listed in red; in orange are mutations that have relative resistance compared to native *BCR::ABL*. Of note, even though both T315I and E255K/V mutated *BCR::ABL* is less sensitive to ponatinib compared to native *BCR::ABL*, these mutations alone do not generally confer resistance to ponatinib in the clinical setting. Rather, resistance is conferred when both mutations are present in compound configuration. Reprinted with permission from Braun TP, Eide CA and Druker BJ. Response and Resistance to *BCR::ABL1*-Targeted Therapies (Source: *Cancer Cell*. 2020; **37**(4): 530–42. doi: 10.1016/j.ccell.2020.03.006.)

either nilotinib or dasatinib, or who had the T315I mutation; ~30% of myeloid BP and ~30–50% of lymphoid BP patients achieved complete haematological response and cytogenetic response with ponatinib treatment. However, median duration of response is only ~5 months.

Fertility preservation and pregnancy

The long-term effect of TKIs on prospective fertility is not known. An increasing number of children had been fathered by male patients receiving imatinib, dasatinib, nilotinib and bosutinib without increased congenital or developmental abnormalities compared to the general population. There is insufficient data regarding ponatinib and asciminib to know whether fathering children is safe for male patients receiving these drugs. In contrast, effective contraception is advised for female patients receiving any of the TKIs as they are all associated with a higher risk of birth defects.

Female patients wishing to conceive electively should ideally achieve and maintain a deep molecular response to allow a trial of cessation, using standard TFR criteria, that will allow for conception. The appropriate washout period between TKI cessation and attempting to conceive is unknown, though pharmacokinetic data would suggest a week should suffice. Regular haematological and molecular monitoring during this period and the subsequent pregnancy is advised. TKIs should be restarted postpartum for patients with loss of molecular response. Should patients require treatment while still pregnant (e.g. loss of complete haematological response with moderate or marked leucocytosis and/or thrombocytosis) interferon-α would be a reasonable choice in the second or third trimester. Regular leucapheresis may be an alternative. Data regarding the use of hydroxycarbamide (hydroxycarbamide

hydroxyurea) in this setting are scant and busulfan should be avoided. Cases also exist where imatinib had been used successfully in the second and third trimester, though experience here is again limited. TKIs should be stopped in women who accidentally conceive while taking TKIs, though the course of action should be individualised. Breastfeeding is contraindicated while on TKI treatment.

Haemopoietic stem cell transplantation

Prior to the introduction of TKIs, CML (especially CML-CP) was one of the most common indications for allogeneic HSCT. Although optimised protocols for HSCT in CML-CP can result in transplant-related mortality as low as ~5–10%, TKIs are still a better option for the vast majority of these patients. Consequently, HSCT is now usually reserved for patients with advanced phase disease or therapeutic failure to all available TKIs.

Allogeneic HSCT outcomes may be predicted using the EBMT risk score, which includes variables such as patient age, stage of disease, donor type (related versus unrelated), stem cell source (PB versus BM) and length of diagnosis (>12 versus <12 months). The bulk of transplant outcome data came from patients who underwent myeloablative conditioning, which usually consists of high-dose cyclophosphamide and either busulfan or total body irradiation. The experience with reduced-intensity conditioning in CML had been steadily increasing and is preferred for older patients.

Problems common to HSCT in other diseases are also applicable in CML. Transplant-related mortality, graft-versus-host disease and relapse-related mortality are significant problems. Many centres will use TKI therapy post allogeneic HSCT and in cases where there is evidence of residual leukaemia by molecular monitoring, though the role for TKIs in this setting has not been fully evaluated. Unlike CML-CP patients, bone marrow RQ-PCR may be a more sensitive marker for minimal residual disease in CML-AP/BP. Donor lymphocyte infusions may be of benefit in patients with disease relapse post-transplant. However, exacerbation of GVHD may be a concern.

Atypical CML/chronic neutrophilic leukaemia

Occasionally patients present with a blood picture suggestive of CML, but the Philadelphia chromosome and the *BCR::ABL1* fusion gene are both undetected (see also Chapters 10 and 35). After exclusion of cryptic variant t(9;22) and atypical *BCR::ABL1* transcripts, a diagnosis of either atypical chronic myeloid leukaemia (aCML, now classified as MDS/MPN with neutrophilia in the current WHO classification) or chronic neutrophilic leukaemia (CNL) may be appropriate.

Atypical CML is an overlap syndrome with features of both myeloproliferative and myelodysplastic disorders. Patients usually present with leucocytosis, with a predominant increase in neutrophils and their precursors. Maturation is dysplastic, and in contrast to CML and chronic myelomonocytic leukaemia (CMML), basophilia, monocytosis and eosinophilia are rare. Patients with aCML fare poorly and often die from marrow failure or transformation to AML.

Like CML and aCML, CNL is characterised by leucocytosis with a predominant neutrophilia. However, unlike aCML, neutrophil morphology is normal in CNL. Neutrophil precursors such as metamyelocytes, myelocytes and promyelocytes are <10% of circulating cells in CNL, which differentiates this disease from CML. The course of CNL is variable, though neutrophilia is usually progressive, accompanied by cytopenia of other lineages resulting in marrow failure, or transformation to AML.

Recurrent genetic lesions have been found in the *CSF3R* and *SETBP1* genes in a number of CNL and aCML patients. The *CSF3R* T618I activating mutation is present in ~80% of CNL cases and forms a criteria for diagnosis. *CSF3R* T618I driven disease is sensitive to ruxolitinib which may be appropriate treatment in selected cases. Mutations in *CSF3R* at other loci are implicated in severe congenital neutropenia. In contrast to CNL, the mutation profile of aCML is more variable. Genes mutated in other myeloid malignancies, such as *SETBP1, ASXL1, N/K-RAS, SRSF2* and *TET2* are commonly observed in aCML. Mutations in *CBL, CSF3R, JAK2* and *ETNK1* are observed but less frequently encountered. The *CSF3R* mutations seen with aCML include the proximal membrane mutation T640N, as well as nonsense and frameshift mutations that result in truncation of the cytoplasmic tail of *CSF3R* (e.g. D771fs, S783fs, Y752X and W791X).

Future prospects

Although the treatment outcome for CML patients has dramatically improved in the last decade, a number of challenges remain. Early transformation to CML-BP remains the outstanding cause of CML-related death. A number of emerging biomarkers with prognostic significance are currently under investigation, and early identification of patients who are at high risk of transformation should become possible. Perhaps more challenging will be the task of finding effective therapies for high-risk patients thus identified and in patients with *de novo* CML-BP. This may rely on the clinical development of small molecule inhibitors with novel mechanisms of action and synergistic activity against *BCR::ABL1*+ cells (Figure 33.13). Advances in allogeneic HSCT, such as continuing decreases in transplant-related mortality and improvements in supportive care, may also be important for this group. At the other end of the spectrum, an increasing number of patients achieve stable and deep molecular responses. Selection of patients likely to be able to stop therapy without relapse, finding the optimal consolidating therapy ahead of a second TFR attempt in patients who have already had one unsuccessful attempt at TFR, as well as strategies to boost the overall TFR rate are all increasingly important priorities for future research.

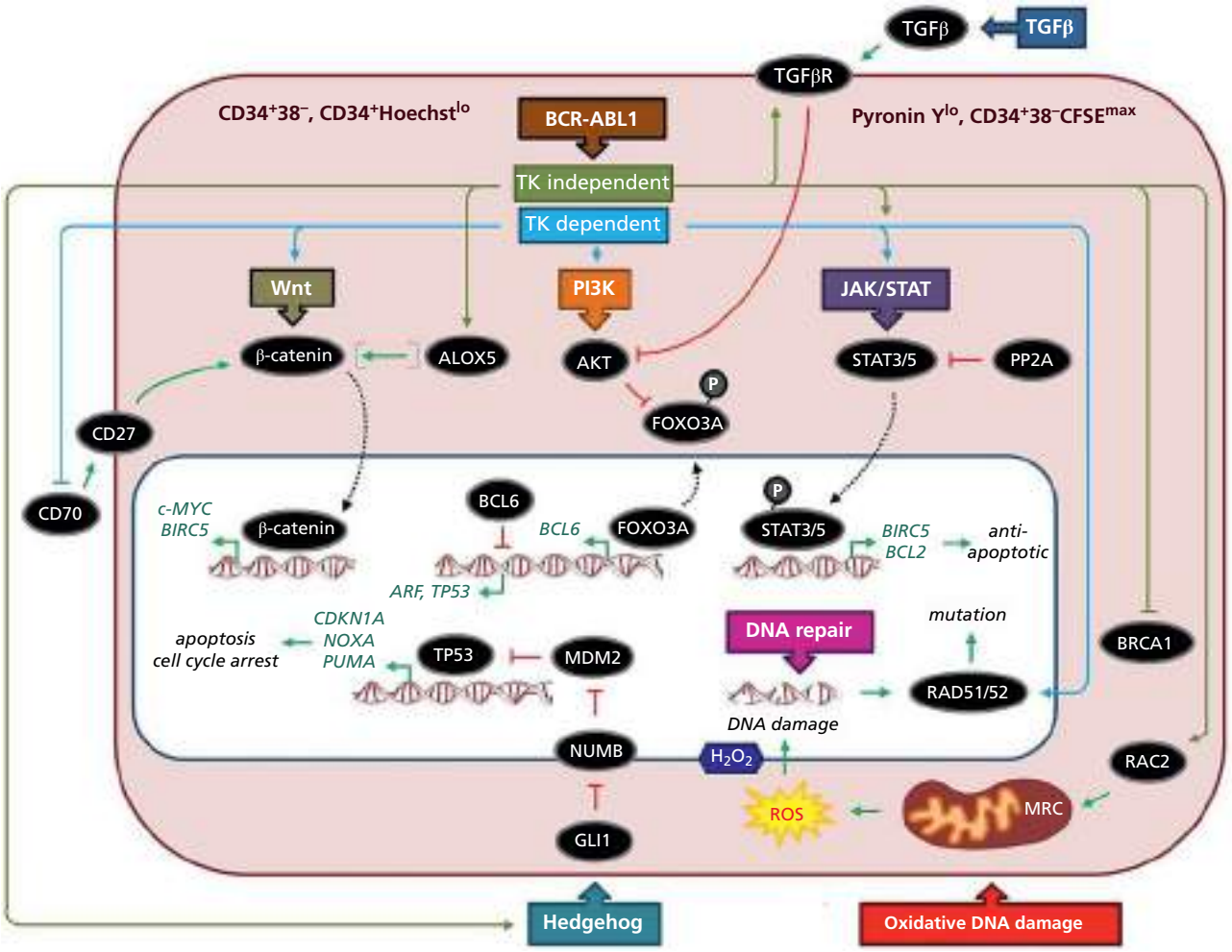


Figure 33.13 A number of critical pathways contribute to CP CML leukaemic stem cells being quiescent and refractory to apoptosis, persistent even with *BCR::ABL*1 inhibition from TKI treatment and prone to DNA damage. They may be identified immunophenotypically as CD34+CD38− Ph+ cells. Targeting some of these signals may be critical to improving therapeutic success in advanced phase CML, as well as patients with failure to achieve treatment-free remission. A number of small molecule inhibitors with activity against some of these targets are already in clinical use, such as ruxolitnib against JAK2 (Source: From Holyoake and Vetrie (2017) *Blood* **129**(12): 1595–606.)

Acknowledgement

The authors would like to thank Dr. Naranie Shanmuganathan for critically reviewing this chapter.

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CHAPTER 34

The myelodysplastic syndromes

34

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Introduction

The myelodysplastic syndromes (MDS) constitute a spectrum of myeloid malignancies with variable degrees of cytopenia and morphologic dysplasia and with a considerable risk for progression to acute myeloid leukaemia (AML). MDS cancer cells display profound molecular and biological heterogeneity leading to different clinical features and propensities for clinical stability or progression. Understanding disease biology is thus key for understanding the disease. MDS patients present with a large variety of disease manifestations from very indolent to frankly aggressive. Clinical management therefore necessitates knowledge about a broad spectrum of diagnostic, prognostic and therapeutic procedures.

Although MDS encompasses a number of heterogeneous subcategories, they share a common origin in the hemopoietic stem and progenitor cell (HSPC). The degree of cytopenia partly defines the WHO subcategories but certain MDS and mixed MDS/MPN subgroups may also present with increased white blood cell, monocyte and platelet counts. Moreover, a diagnosis of MDS can be made in patients with mild or borderline anaemia if definitive morphologic or cytogenetic findings are present.

The most recent WHO Classification from 2022 encompasses MDS, mixed MDS-myeloproliferative syndromes (MDS MPN), secondary myeloid neoplasms and MDS of childhood. This chapter will cover all these entities, in the general sections of this chapter termed 'MDS'.

History and classification

Patients who today are diagnosed as MDS were for decades considered a diagnostic challenge. The disease features were dominated by treatment-refractory cytopenias and patients often had a dismal outcome. The high propensity of leukaemic progression initially led to the term 'preleukaemia' being coined in 1953. Haematopathological studies revealed morphological abnormalities that with time were identified as dysplastic features and which were considered either pathognomonic or supportive for the diagnosis. The bone marrow frequently showed an increased percentage of myeloblasts, but not to the extent that the criteria for AML were met. Due to the somewhat subjective nature for defining bone marrow dysplasia, it took several international consensus meetings before MDS was recognised as a separate entity within the first French-American-British (FAB) MDS classification, published in 1982. This classification divided patients with cytopenia and bone marrow dysplasia into five categories; those without excess of myeloblasts with or without the presence of ring sideroblasts, two groups with excess of myeloblasts 5–19 and 20–29%, respectively, and finally patients with monocytosis, termed chronic myelomonocytic leukaemia, CMML. The subsequent WHO classification was first published in 2001 and contained two major changes; the boarder against acute myeloid leukaemia was lowered from 30 to 20% bone marrow myeloblasts, and the '5q- syndrome' was introduced as a separate entity. Moreover, the overlap condition mixed myelodysplastic myeloproliferative syndromes (MDS/MPN) was introduced as a separate category. The WHO classification

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).

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Table 34.1 The WHO 2016 classification of MDS and MDS/MPN.

Myelodysplastic/ myeloproliferative neoplasms (MDS/MPN)	Myelodysplastic syndromes (MDS)
Chronic myelomonocytic leukaemia (CMML)	MDS with single lineage dysplasia
Atypical chronic myeloid leukaemia (aCML), *BCR::ABL12*	MDS with ring sideroblasts (MDS-RS)
Juvenile myelomonocytic leukaemia (JMML)	MDS-RS and single lineage dysplasia
MDS/MPN with ring sideroblasts and thrombocytosis (MDS/ MPN-RS-T)	MDS-RS and multilineage dysplasia
MDS/MPN, unclassifiable	MDS with excess blasts
	MDS with isolated del(5q)
	MDS, unclassifiable
	Provisional entity: Refractory cytopenia of childhood
	Myeloid neoplasms with germ line predisposition

has been revised both in 2008, 2016 and 2022. An alternative classification system has also recently been proposed (International Consensus Classification of Myeloid Neoplasms and Acute Leukaemias), which broadly parallels the WHO system, and for the purposes of this chapter, the WHO classification system will be used. The classification systems stimulated the development of international prognostic scoring systems (IPSS) for patients with MDS, the initial publication in 2007, the revised IPSS (IPSS-R) in 2013, and recently the molecular IPSS-M. These will be described below. Table 34.1 shows the WHO 2022 classification of MDS and MDS/MPN.

Demographics and clinical presentation

Information about the incidence of MDS is based on large academic registries and population-based national cancer registries. Recent population-based studies report median ages of presentation of 75–76 years. European national registries report annual incidences of approximately 3–4 per 100,000. Irrespective of whether population-based or academic, all registries show a sharp increase of incidence with age, making MDS and its related disorders common blood cancers in the elderly population.

At diagnosis, the majority of patients with MDS have symptoms related to cytopenia, and these usually increase with time. Anaemia is the most frequently occurring cytopenia. According to a large population-based registry 11 and 42% of newly diagnosed patients had Hb levels <8 and 8–10 g/dL, respectively, and 50% needed erythrocyte transfusions, 40% had platelet counts below 100×10^9/L, 5% received platelet transfusions and 20% had neutrophil counts $<0.8 \times 10^9$/L. Hence, symptoms of anaemia dominate the initial clinical picture, while bleeding complications and infections become more pronounced during the course of disease.

A portion of MDS patients present with systemic inflammatory and autoimmune diagnoses (SIAD) before, in conjunction with, or after the diagnosis of MDS. Systemic vasculitis is reported in 32%, connective tissue disease in 25%, inflammatory arthritis in 23%, and neutrophilic disorders in 10% of these cases. There is a significant association between CMML and systemic vasculitis, which will be further discussed below. Other manifestations encompass fever, skin abnormalities including Sweet syndrome, and bleeding due to dysregulated coagulation. It is important to recognise the MDS diagnosis in these patients, as intervention with corticosteroids or azacitidine may relieve symptoms.

Studies on the quality of life (QoL) in MDS uniformly show a significant impact of disease on QoL and everyday life. Anaemia is strongly associated with fatigue, dyspnea and reduced exercise capacity, and pancytopenia adds on a risk of infection, bruising and bleeding. Severe MDS implies frequent transfusions leading to high dependency on the health care system and patients often feel that they are 'on a short leach'. Treatment of higher-risk MDS also necessitates frequent and regular visits for long periods, even in case of successful outcome. To live with the anticipated risk of disease, progression is associated with significant anxiety. An American study showed that the three most frequently reported symptoms were fatigue, pain and worry. A prospective European Registry study comparing patients with lower-risk MDS with an age- and sex-matched reference population reported moderate/severe problems in the dimensions of pain/discomfort (50%), mobility (41%), anxiety/depression (38%) and usual activities (36%). Limitations were more frequent in older patients, in females, and in those with high comorbidity burden or red blood cells transfusion need.

Aetiology

The cellular origin of MDS lies within the HSPC compartment and can usually be tracked back to the multipotent haematopoietic stem cell. Disease-propagating cells accumulate in the bone marrow as a result of a complex interplay between genetic and epigenetic alterations, the bone marrow microenvironment, the immune system and exogenous factors. It has recently been shown that this process can develop over several years and even decades.

Germline predisposition to myeloid neoplasms

MDS was previously considered a malignancy only rarely associated with germline predisposition. Exceptions were childhood conditions and syndromes, such as Diamond–Blackfan anaemia, Fanconi anaemia, Shwachman–Diamond syndrome and Kostmann´s disease, which will be described below in the

section about paediatric MDS. Another group of inherited MDS are the rare congenital sideroblastic anaemia (CSA) caused by mutations in genes involved in the iron and mitochondrial pathways. The most common of these are caused by germline mutations in *ALAS2,* followed by *SLC25A38, GLRX5, SLC25A38, HSPA9, HSCB, ABCB7* and others. The latter is interesting since the *SF3B1* mutations underlying acquired MDS with ring sideroblasts lead to miss-splicing and marked downregulation of *ABCB7*.

With the rapid development of genomics in haematology, it has become increasingly clear that also adult myelodysplastic syndromes can develop on the basis inherited mutations in germline DNA. Individuals with germline predisposition exhibit an increased risk to develop of myeloid neoplasms, mainly AML and MDS. Estimates suggest that between 5 and 10% of patients with MDS or AML carry pathogenic germline variants.

Myeloid neoplasms (MNs) with germline predisposition were recognised in a separate entity of 'Secondary Myeloid Neoplasms' in the WHO 2022 Classification. Germline mutations are divided into those predisposing to MN without a pre-existing disorder, mutations with pre-existing platelet dysfunction and mutations associated with organ dysfunction. *GATA2* and *RUNX1* mutations are relatively common and mandate continuous surveillance of asymptomatic carriers, due to the high risk of developing MN. Mutations in the telomerase complex usually imply a complicated clinical presentation with multi-organ involvement including lung and liver failure, in addition to a high risk to develop bone marrow failure or myeloid neoplasms. Heterozygous mutations in the *SAMD9* and *SAMD9L* genes comprising both loss and gain of function are associated with a range of haematological, immunological and neurological symptoms. These genes are located on chromosome 7 and render a high risk of progression to monosomy 7 MDS. Interestingly, development of monosomy 7 or 7q- may be associated with deletion of the mutated allele and reversion of the haematological features. More recently, identified homozygous mutations in *ERCC6L2* were shown to predispose to the development of somatic *TP53* mutations and severe MDS or AML. Recently, heterozygous mutations in *DDX41* (DEAD-box Helicase 41) were described as one of the most common predisposing mutations in MDS, probably present in as much as 3% of unselected adult patients. *DDX41* germline mutations predispose to MN at higher ages than most other predisposing mutations, making it an important gene to analyse in potential adult sibling donors. A summary of germline predisposition genes and subsequent diseases is found in Table 34.2.

Identification of a germline predisposition in a patient with MDS can be of crucial clinical significance. It may tailor therapy and dictate the selection of donor and conditioning regimen for allogeneic HSCT. It may also, depending on the risk for MN associated with a specific mutation, imply a recommendation to investigate first-degree relatives and to offer relevant regular controls for healthy carriers to allow for early intervention if MN develops. Several collaborative groups have recently published practical guideline programmes for diagnosis and management of germline conditions. However, new knowledge will likely be presented over the next years.

Myeloid neoplasms post cytotoxic therapy

Treatment with chemotherapeutic agents or irradiation for another primary cancer or other medical condition can induce genetic damage to the HSPC compartment and subsequent development of MDS or AML, in the WHO 2022 classified as myeloid neoplasms post cytotoxic therapy (MN-pCT). Large epidemiological studies indicate that around 10% of adult MDS patients should be classified as MN-pCT. The current WHO classification group all MN-pCT in one category, irrespective of the percentage of myeloblasts or cytogenetic features, and of the time between previous treatment and diagnosis of MDS. This may be a useful simplification but becomes less relevant when an elderly patient develops i.e. MDS-RS decades after a previous treatment for cancer. Altogether, patients with MN-pCT have a shorter expected overall survival than patients with *de novo* MDS with corresponding risk categories, but MN-pCT usually display a higher degree of complexity with regard to chromosomal and mutational patterns. It is likely that future classifications and risk scores for tMN to a higher extent will include mutational profiles. With few exceptions, MN-pCT should if possible be considered for curative therapeutic regimens.

MDS developing from clonal haemopoiesis of indeterminate potential

Clonal haemopoiesis of indeterminate potential (CHIP) is a relatively new field within haematology and will be described elsewhere in the textbook. CHIP may be present in the absence of cytopenia but may also co-exist with cytopenia without fulfiling the criteria for MDS (clonal cytopenia of unknown significance, CCUS). While some large epidemiological studies have shown that individuals with CHIP have a higher risk of developing cardiovascular disease and myeloid neoplasms, others have failed to show a strong correlation. The most frequent CHIP mutations encompass *TET2*, *DNMT3A* and *ASXL1,* but mutations in several other genes have also been described. Recent studies using next-generation sequencing tools allowing for detection of clones down to 1–2% show that close to 50% of individuals above 70 years carry CHIP clones, but whether such clones predispose for MN or simply should be viewed as normal ageing is not yet known. The frequent finding of CHIP in middle-aged and elderly individuals may also add to the understanding of MN-pCT; silent haematopoietic clones may be resistant to the cytotoxic exposure for the primary cancer and have the potential to undergo selective expansion and transformation.

Table 34.2 Germline mutations predisposing to myeloid neoplasms. Within each category, genes are listed in alphabetical order. Estimated risks for myeloid neoplasm are not given as the literature report large variations.

Disease/gene	Comment
*Germ line mutations predisposing to myeloid neoplasms**	
ANKRD26	Adults. Pre-existing thrombocytopenia
CEBPA	Children-adults. Mainly AML
DDX41	Adults. Secondary acquired *DDX41* mutation
ETV6	Adults. Pre-existing thrombocytopenia. Mainly ALL
GATA 2	Children-adults. Bone marrow failure common
RUNX1	Children-adults. Pre-existing thrombocytopenia
SAMD9/SAMD9L	Children-adults. Immune defects, neurological manifestations
TET2	Only rare families reported
*Classical inherited bone marrow failure syndromes***	
Fanconi anaemia	Children. More than 20 FA genes reported
Schwachman Diamond syndrome	Children-adults. High risk of somatic *TP53* mutations
Severe congenital neutropenia	Children-adults. *ELANE*, *G6PC3*, *HAX1*, *JAGN1* and others
Diamond Blackfan anaemia	Children. Frequent craniofacial, urogenital malformation and cardiac defects. Ribosomal genes *RPS19*. Rare cases *GATA1*
Telomere biology disorders	Children-adults. *DKC1*, *TERT*, *TERC* and several other genes. Variable presentation, often multi-organ failure
Down syndrome	
Trisomy 21	Children. Mainly AML
DNA repair syndromes and TP53	
ERCC6L2	Adults. BM failure and high risk of somatic *TP53* mutations
Li Fraumeni syndrome	Children-young adults. Constitutional *TP53*. Solid tumours and leukaemia.
RAS pathway mutations	
JMML in Neurofibromatosis type 1 (NF1) syndrome & CBL syndrome	Germline RAS pathway mutations and acquired biallelic inactivation of the gene
Noonan syndrome with myeloproliferation	Young children. RAS pathway mutation with myeloproliferation. Spontaneously resolves <1 year of age

Bloom syndrome, an autosomal recessive disorder with BLM mutation leading to genomic instability and propensity for early cancer, may be mentioned but leads to a broad range of cancer including but not enriched for leukaemia and lymphoma. Note that JMML most frequently is associated with somatic mutations in RAS pathway genes, only JMML associated with NF1 is associated with germline RAS mutations.
* Lymphoid neoplasms also reported
** usually with multi-organ engagement
(*Source:* Sahoo *et al.* 2020.)

However, it is clear that MN-pCT can develop also in children and adults without pre-existing CHIP.

Age

The most significant risk factor for MDS is age, but whether age itself should be considered an aetiological factor is not confirmed. The incidence of MDS increases sharply from 70 years and onwards, and the curve is different from other myeloid diseases such as acute and chronic myeloid leukaemia which are more frequent also in younger adults. CHIP could be one reason for the increasing incidence, but unknown changes in the immune system and in the bone marrow microenvironment also contribute.

Pathogenesis

The mechanisms by which MDS emerges are not fully understood but modern techniques such as NGS, gene expression analyses, epigenetic studies of DNA methylation and histone modifications as well as new possibilities of cell sorting and imaging have greatly increased our knowledge. Several dysfunctional intracellular pathways have been identified in the MDS cells e.g. aberrant RNA splicing, epigenetic regulation, ribosomal function and apoptosis. In addition to the intrinsic factors, it has been demonstrated that the immune system is dysregulated and most likely contributes to disease features. Disturbances in the bone marrow niche are another extrinsic factor with a potential pathogenetic role (Figure 34.1).

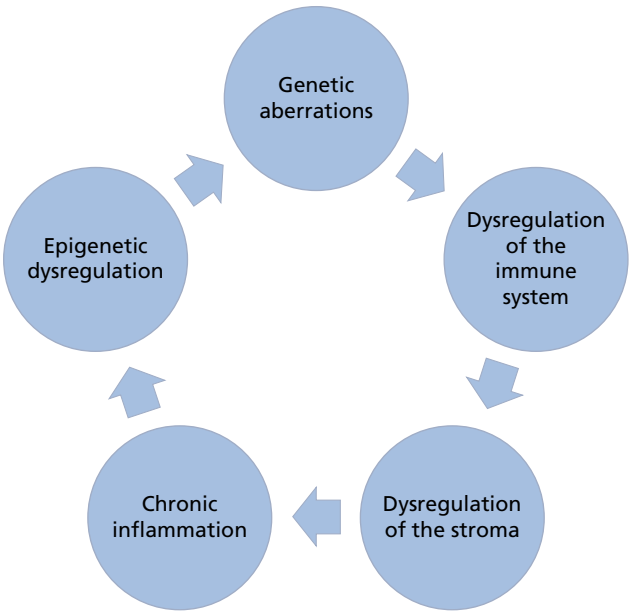

Figure 34.1 Schematic overview of pathways contributing to the pathogenesis of MDS and MDS/MPN.

Stem cells and differentiation

An important feature in MDS is the disturbed haemopoietic differentiation. In contrast to acute leukaemia where a differentiation block prevents cells from maturing, there is usually no complete differentiation block in MDS. MDS progenitors may differentiate into mature blood cells able to enter the circulation, but the maturation process is ineffective resulting in a lack of functional cells in the blood. This disturbed differentiation is reflected by the characteristic morphological dysplasia of bone marrow precursors.

The lack of effective differentiation may lead to progressive expansion of the immature cell compartment. In higher-risk patients, this is reflected as a morphological increase of marrow blasts but also in lower-risk patients without morphological increase of marrow blasts there may be an expansion of HSPC.

Modern cell sorting and sequencing technologies have allowed the identification of driver mutations in HSPC from MDS bone marrow samples. Using xenograft models, patient-derived stem and progenitor cells have been demonstrated to establish the MDS disease following transplantation into immune-deficient mice. While data from higher-risk MDS is more ambiguous, studies of lower-risk MDS provide evidence that the disease is initiated in rare multipotent haematopoietic stem cells and propagated to progenitor and precursor cells further down in the differentiation tree. There is thus a gradual expansion of a new parallel haematopoiesis which over time will dominate the bone marrow at the expense of the increasingly suppressed original haematopoiesis. The fact that MDS clearly is a disease that is propagated from the stem cell compartment, provides an explanation why MDS is so difficult to cure by chemotherapy only and why stem cell targeting therapy e.g. allogeneic stem cell transplantation is needed for complete cure.

Apoptosis

There is an expansion of immature cells in the bone marrow parallel to a reduction in the number of mature cells entering the blood. An important feature mediating this paradoxical phenomenon is increased apoptosis (programmed cell death) of haematopoietic precursor cells, which is more prominent in lower-risk MDS than in advanced MDS. Apoptosis can be initiated extrinsically by various cytokines, notably tumour necrosis factor (TNF)-α, Fas-ligand and TNF-related apoptosis-inducing ligand (TRAIL) that are frequently upregulated in MDS, serving as negative regulators of haematopoiesis. In addition, extrinsic stimuli may involve cytotoxic T cells and signals from bone marrow stromal cells, likely via activation of similar pathways. Apoptosis may also be activated through intrinsic pathways activated by DNA damage and mitochondrial dysfunction leading to cytochrome *c* release and caspase activation.

Genetics

The genetic landscape of MDS is today very well delineated thanks to novel technologies that have allowed a detailed characterisation of the MDS genome. Early studies identified structural chromosomal aberrations (e.g. del 5q and monosomy 7) using metaphase karyotyping, FISH and later SnP-arrays. Later, next-generation sequencing (e.g. whole genome sequencing, whole exome sequencing and targeted gene panels) have also allowed the identifications of point mutations.

Chromosomal aberrations

Around 50% of patients have structural chromosomal aberrations which can be detected using conventional karyotyping (G-banding). The frequency of cytogenetic aberrations is lower in MDS/MPN and CMML (around 30%). In contrast to AML where aberrations are dominated by balanced translocations (e.g. t[8;21] and t[15;17]), aberrations seen in MDS and MDS/MPN are usually unbalanced (e.g. del 5q, monosomy 7 and del 20q) resulting in copy number abnormalities (CNA). Changes can appear as single aberrations or part of a complex karyotype (≥3 aberrations). A complex karyotype is often accompanied by *TP53* mutations and associated with very poor prognosis. Single-nucleotide polymorphism arrays (SnP-arrays) can detect smaller abnormalities (down to <20 k base pairs) that are not detected by standard karyotyping. SnP-arrays can also detect copy-neutral loss of heterozygosity (LOH) or uniparental disomy (UPD).

Somatic mutations

Whole-genome, whole exome and targeted panel sequencing have allowed a detailed characterisation of the MDS genome and identification of somatic heterozygous driver mutations. MDS patients have on average 2–3 driver mutations in the coding region, with a range from 0 to 10. This is significantly fewer mutations compared to many solid cancers but more than in some other haematological cancers such as chronic myeloid leukaemia. Around 50 genes have been reported to be recurrently mutated in MDS, with highly recurrent mutations shown in Table 34.3. Patients with higher-risk MDS disease as well as patients with MDS/MPN including CMML, have on average more mutations compared to patients with lower-risk MDS. Importantly, the number of mutations is in itself a prognostic factor and is included in the recently published prognostic score IPSS-M.

Driver mutations identified in MDS can be categorised into functional groups, each group providing a distinct contribution to disease pathogenesis. The most commonly mutated groups of genes encompass the spliceosome, DNA methylation, histone acetylation, transcription factors and genes involved in signalling (Figure 34.2).

Combinations of mutations and chromosomal aberrations are sometimes enriched, such as *SF3B1* mutation with either *JAK2, TET2* or *ASXL1*, and complex karyotype with *TP53*, whereas other mutations seem to be mutually exclusive e.g. spliceosome and cohesin factor mutations, and *TET2* with *IDH1* or *IDH2*.

The spliceosome mutations are more common in MDS than in any other myeloid neoplasm and probably cause several of the dysplastic features. These mutations contribute to pathogenesis through the resulting downstream RNA missplicing of specific genes. Different spliceosome mutations lead to completely different disease features; *SF3B1* to ring sideroblasts (MDS-RS), SRSF2 to monocytic dysplasia, and *U2AF1* to leukaemic transformation.

Mutations in genes involved in DNA methylation and histone modification (epigenetic regulation) e.g. *TET2, DNMT3A, IDH1/2, EZH2* and *ASXL1* are found in up to 60–70% of all

Table 34.3 Somatic mutations in MDS and MDS/MPN. Colour code represent the percentage of patients with a specific disease harbouring the mutation, where white colour represents 0–1% mutation frequency, very light blue 2–5%, light blue 5–15%, blue 16–24%, dark blue 25–40%, very dark blue 41–70% mutated, and night dark blue >70% mutation frequency.

	MDS-RS	All MDS	CMML	MDS/MPN-U	JMML	aCML	MDS/MPN-RS-T
ASXL1							
CALR							
CBL							
CEBPA							
DNMT3A							
ETV6							
EZH2							
FLT3							
IDH1							
IDH2							
JAK2							
KIT							
KRAS							
MPL							
NF1							
NRAS							
PTPN11							
RUNX1							
SETBP1							
SF3B1							
SRSF2							
TET2							
U2AF1							
ZRSR2							

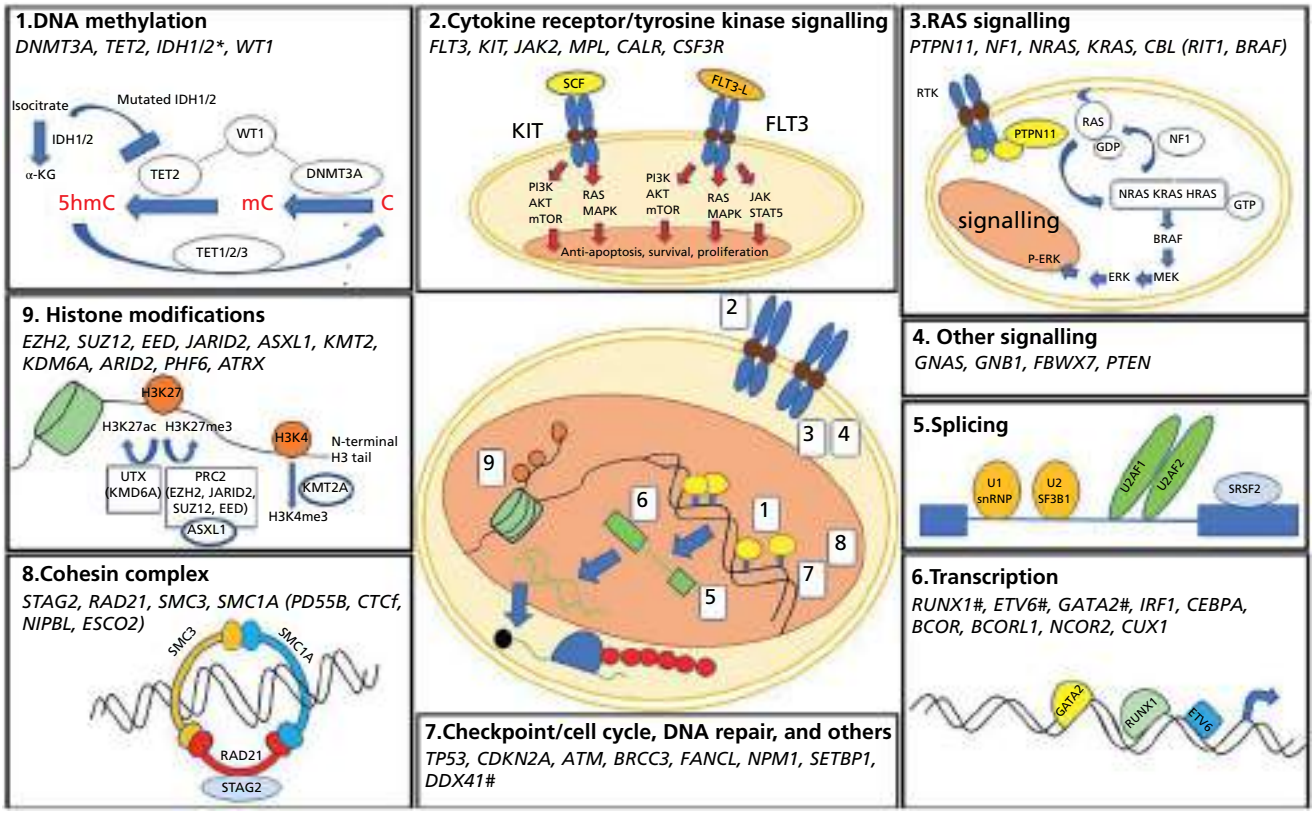

Figure 34.2 Major driver genes and their pathways in MDS and MDS/MPN. The numbers 1—9 in the central figure represent the cellular location of the respective pathway 1—9 described in the outer circle of figures. *mutaded IDH1 and *IDH2* but not the unmutated forms, affect DNA methylation. #germline variants are implicated in the predisposition to MDS and AML (Source: Gene list and pathways adapted from Ogawa S, *Blood* 2019.)

MDS patients (Table 34.3 and Figure 34.2). *TET2* and *DNMT3A* are also the most common mutations in CHIP individuals and may constitute early events in MDS pathogenesis.

Mutations in *TP53* are, as in cancer in general, associated with poor prognosis. *TP53* mutations are frequently found in association with complex karyotypes, but can also be associated with certain germline mutations, and with isolated del(5q), as discussed below. Importantly, a recent large study shows that mainly bi-allelic *TP53* mutations, i.e. two or more mutations, or deletion of the wild-type allele by loss of heterozygosity (LOH) or concomitant 17p deletion have a deleterious impact on prognosis and this is recognised in the WHO 2022 classification as a separate entity. This adds a challenge to diagnostic DNA sequencing in clinical routine.

Genes involved in the cohesin complex are mutated in 10–15% of MDS patients. Examples of cohesin genes are *STAG2*, *RAD21* and *SMC1A* and mutations in these genes are mutually exclusive (Table 34.3, Figure 34.2). The cohesin complex regulates large chromosomal formations and is probably important for regulating the access of transcription factors to the DNA.

RUNX1, GATA2, ETV6 and BCOR are transcription factors that likely directly interact with the differentiation process. Interestingly, several of these also appear as germline mutations (Tables 34.2 and 34.3, Figure 34.2).

Genes coding for proteins involved in signalling e.g. RAS-pathway mutations, *JAK2, KIT, CBL* and *MPL* are regulators of proliferation and more commonly mutated in MDS/MPN than in MDS. Mutations in these genes result in hyperproliferative haematopoiesis. RAS-pathway-driven hyperproliferation is relatively common in childhood MDS and related disorders (Tables 34.2 and 34.3, Figure 34.2).

Epigenetics

The epigenome consists of DNA methylation and histone modifications. The MDS epigenome exhibit, as cancer genomes in general, global hypomethylation and promoter hypermethylation. The characteristics of the MDS epigenome may in part be explained by mutations in epigenetic regulators as described above; however, the dysregulated epigenome can also be a consequence of stochastic epigenetic drift seen with increasing age where cells gradually diverge from each other. Potentially, environmental factors e.g. chronic inflammation may facilitate the epigenetic drift.

Immune dysregulation

Immunological abnormalities are commonly encountered in MDS, suggesting that they may play a role in the pathogenesis of the disease. This is particularly apparent in cases of hypoplastic MDS that may have features in common with aplastic anaemia. A hypocellular bone marrow is seen in approximately 10% of MDS patients and is recognised as a distinct entity in WHO 2022 (hypoplastic MDS) and may indeed represent one end of the MDS spectrum that biologically resembles or overlaps with aplastic anaemia. The other main reason for hypoplastic MDS is bone marrow failure caused by germline mutations, as previously discussed. Hypoplastic MDS is characterised by a pathological immune response, resulting in autoimmune destruction of normal HSPCs. In addition, acquired mutations in the PIGA gene characteristic of paroxysmal nocturnal haemoglobinuria (PNH) may develop, presumably through expansion of small clones in an otherwise hypoplastic marrow, suggesting that PNH is part of this spectrum. Conversely, dysplastic clones carrying cytogenetic abnormalities or point mutations can emerge in both aplastic anaemia and PNH after treatment and may evolve into (or result in) MDS over time.

Expanded CD8+ cytotoxic T-cell clones can occasionally become neoplastic, resulting in the diagnosis of T-cell large granular lymphocytic leukaemia (T-LGL) that also falls into the overlap spectrum of bone marrow failure states (Figure 34.3). T-LGL clones have been demonstrated in a relatively large proportion of MDS at diagnosis, but do not seem to influence prognosis, clinical picture or response to treatment. In addition to clonal T-cells, it is also described that MDS patients may have separate plasma cell clones (monoclonal gammopathy of undetermined significance) and sometimes overt myeloma.

A role for the immune system in the pathogenesis of MDS and MDS/MPN is also indicated by the high incidence of autoimmune disease of 10–25%. Patient with MDS/MPN and in particular CMML have a high incidence of inflammatory symptoms e.g. arthritis, vasculitis and skin lesions, compared to age-matched controls. However, the most persuasive evidence for immune dysregulation in MDS arguably comes from the recognition that patients with some forms of the disease, notably hypocellular MDS, like aplastic anaemia, respond well to immunosuppressive therapy with antithymocyte globulin and/or cyclosporin resulting in durable haematological recovery and abrogation of T-cell clones.

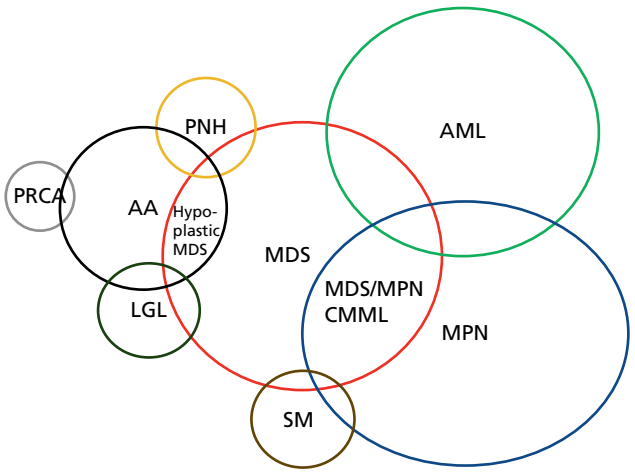

Figure 34.3 Myeloid neoplasms depicting the overlap between disease entities. AML acute myeloic leukaemia, MDS myelodysplastic syndromes, MPN myeloproliferative neoplasms, CMML chronic myelomonocytic leukaemia, SM systemic mastocytosis (can occur with another myeloid disease frequently MDS or MPN, most frequently CMML, T-LGL T-cell large granular lymphocytic leukaemia), PNH paroxysmal nocturnal haemoglobinuria, PRCA pure red cell anaemia, AA aplastic anaemia.

Bone marrow niche dysregulation

The bone marrow microenvironment in MDS frequently shows abnormal features including hypercellularity, abnormal localisation of immature precursors (ALIPs), vascular remodelling and fibrosis. Molecular characterisation of stromal niche cells has revealed genetic, epigenetic and transcriptional alterations. Co-culture models also indicate that MDS niche cells are functionally abnormal, supporting MDS but not healthy HSPC. The bone marrow niche in MDS is pro-inflammatory with increased concentrations of pro-inflammatory cytokines e.g. TNF, IL-6 and S100A8/A9. These factors may induce genotoxic stress, a potential mechanism for malignant transformation.

Specific disease entities within the MDS classification

MDS with low blasts and isolated 5q deletion

MDS with an isolated deletion of the long arm of chromosome 5 (MDS-5q) was early recognised as an MDS entity with specific clinical features. Patients present with severe markedly macrocytic anaemia, hypoplastic erythropoiesis, typical bone marrow morphology with hypolobated megakaryocytes and slightly elevated platelet counts. The WHO classification accepts one additional karyotypic abnormality as long as chromosome 7 is not involved, the most common being trisomy 8. Haploinsufficiency of casein kinase (*CK1α*, a gene within the common deleted region of del(5q) MDS) appear to be of central importance for the phenotype. The drug lenalidomide induces ubiquitination of *CK1α* by the E3 ubiquitin ligase cereblon, resulting in *CK1α* degradation and sensitivity to lenalidomide. Erythropoietin-stimulating agents (ESAs) should, as for other lower-risk MDS be considered first-line treatment for the anaemia of these patients, but lenalidomide can lead to

complete clinical and cytogenetic remission and is licensed as second-line treatment for del(5q) MDS. However, as HSC show a relative resistance to lenalidomide treatment is not curative and resistance often develops with time. Importantly, 15–20% of 5q- syndrome patient develop *TP53* mutated subclones and as these cells are completely resistant to lenalidomide, mutated cells can be selected during treatment. *TP53* mutated leukaemic transformation of 5q- syndrome is a serious and usually fatal complication and it is recommended that patients should be checked for mutational status before and during lenalidomide treatment and that allogeneic SCT should be considered an option in *TP53* mutated patients. Around 50% of del(5q) deletions in MDS are not associated with a 5q- syndrome, but with a complex karyotype, very high-risk MDS and frequent multi-hit *TP53* mutations. These patients have a dismal prognosis even after allogeneic stem cell transplant.

MDS with low blasts and *SF3B1* mutation (MDS-SF3B1)

MDS-SF3B1 is another genetically-defined MDS subset in the WHO 2022 classification following two pivotal papers published 2011 describing recurrent mutations in splicing factor 3b subunit 1 (*SF3B1*) in more than 80% of patients with MDS associated with ≥5% bone marrow ring sideroblasts (RS) i.e. erythroblasts with iron-filled mitochondrial localising around the nucleus. The remaining MDS with RS are classified as wild-type SF3B1 and ≥15% RS. MDS-SF3B1 patients present with slowly developing macrocytic anaemia, normal or slightly elevated platelet count and typically normal karyotype. The risk for progression to higher-risk MDS and leukaemic transformation is low. Untreated, anaemia will in most cases progress and lead to chronic transfusion need. Before the introduction of erythropoietin treatment and iron chelation, the most common fatal complication for these patients was progressive iron overload and organ failure, but with modern treatment, these patients can survive for decades. *SF3B1* mutations lead to missplicing of RNA with subsequent RNA decay and under-expression of genes involved in erythropoiesis, mitochondrial function and iron transport. The characteristic mitochondrial ferritin accumulation in MDS-RS is associated with missplicing and reduced expression of the iron transporter protein gene *ABCB7,* and mechanistic studies link *ABCB7* expression to defective erythropoiesis. Recent studies have tracked back the *SF3B1* mutations to multipotent haemopoietic stem cells, making MDS-RS a true HSC disease. *SF3B1* is often the only driver mutation, but can also be accompanied by CHIP mutations, primarily *TET2* and *DNMT3A*. Primary treatment of MDS-RS is erythropoietin, which if introduced before the onset of a regular transfusion need can lead to normal or at least un-transfused haemoglobin levels in a high proportion of patients, with a response duration of several years. In addition, increased levels of the transforming growth factor beta (TGFβ) superfamily inhibitors of erythropoiesis can be targeted by luspatercept, a recombinant fusion protein, considered to bind transforming growth factor β superfamily ligands and reduce SMAD2 and SMAD3 signalling, reduce erythroid hyperplasia, and enhance erythroid maturation and haemoglobin levels. Luspatercept was recently approved as second-line treatment for MDS-associated anaemia, with particularly high rates of response in MDS-SF3B1.

Hypoplastic MDS

MDS presenting with severe pancytopenia and hypoplastic bone marrow is another subtype of MDS, however more heterogeneous than 5q- syndrome and MDS-RS. Bone marrow cellularity is lower than the average for the age of the specific patient and often <30%. The median age is lower than for MDS in general. Karyotype is often normal, but abnormalities involving chromosome 7 can also be found. This subtype of MDS may be difficult to differ from aplastic anaemia, and bone marrow biopsy with immunohistochemistry is mandatory, as is next-generation sequencing analysis (NGS). A proportion of patients with hypoplastic MDS show predisposing germline variants including *GATA-1, DDX41* and *SAMD9/SAMD9L* variants, and mutations in telomere genes, but in others, the mechanism seems to be similar to that of aplastic anaemia. Some patients, provided no germline background or adverse genetic aberration can be detected may respond to immunosuppression.

Myelodysplastic myeloproliferative overlap diseases (MDS/MPN)

In the 2022 WHO classification, the MDS/MPN category consists of four specific proliferative disease entities (see Table 34.1); Chronic myelomonocytic leukaemia (CMML), Myelodysplastic/myeloproliferative neoplasm with neutrophilia, Myelodysplastic/myeloproliferative neoplasm with *SF3B1* mutation and thrombocytosis and Myelodysplastic/myeloproliferative neoplasm, not otherwise specified (MDS/MPN-NOS). Juvenile myelomonocytic leukaemia, JMML, is a specific entity described more in detail below in the context of paediatric MDS.

Chronic myelomonocytic leukaemia

CMML is the largest subgroup within the MDS/MPN category, with an incidence of 0.4 per 100 000 inhabitants. Two-thirds of patients are male, and the median age of disease onset is 70 years. The risk of AML transformation is around 15–20% thus less than in other MDS, whereas inflammation and autoimmune disease are frequent, occurring in 20–30% of patients.

For CMML diagnosis, persistent monocytosis >0.5 × 10^9/L and constituting more than 10% of leucocyte count with no other explanation of monocytosis, is required. If monocytes are 0.5-<1 × 10^9/L, dysplasia in at least one lineage as well as an acquired clonal cytogenetic or molecular abnormality, is required for diagnosis.

There are two subtypes of CMML, a myeloproliferative (MD-CMML) with leucocyte count ≥13 × 10^9/L or myelodysplastic (MD-CMML) with leucocyte count <13 × 10^9/L, a nomenclature that originates from the 1994 FAB classification. MP-CMML has a worse prognosis compared to MD-CMML. The recent WHO classification divides CMML into subgroups CMML-1 and CMML-2 based on the combined percentage of blasts plus promonocytes in bone marrow and blood. Overall survival for CMML is 12–24 months; however, some patients may live more than 10 years. The karyotype is normal in 70% of cases, but most patients harbour myeloid mutations, with *TET2* being the most common. Over 85% of patients have at least one mutation in *TET2*, *SRSF2* or *ASXL1* (Table 34.3). There is no specific treatment for CMML, however non-proliferative CMML have been included in hypomethylating trials and have similar response rates as other MDS. Recently, a large retrospective study has shown that there is a survival benefit for HMA treatment in higher blast count CMML. In proliferative CMML, hydroxycarbamide is used. Allogeneic stem cell transplantation is the only potential cure.

MDS/MPN-NOS

The disease entity of MDS/MPN-NOS was first established in the 2001 WHO classification as an MDS/MPD with thrombocytosis and leucocytosis. With the current definition, MDS/MPN-NOS accounts for around 2–5% of myeloid malignancies. The diagnosis requires at least one lineage of dysplasia in combination with myeloproliferation, without fulfiling the criteria for CMML or other MDS/MPN. About 70% of patients have a normal karyotype, and molecularly there is no distinct pattern however *ASXL1* mutations are common, 30–55% of patients, followed by *TET2*, *SRSF2*, *SETBP1* and *JAK2* mutations (Table 34.3). Overall survival is poor, around 12–32 months, and age >60 years, thrombocytosis, and bone marrow myeloblasts over 5% blasts indicate poor outcome.

There are no specific treatment recommendations for MDS/MPN-NOS. Hypomethylating agents are less effective than in MDS, 20–30%. Immunomodulatory regimens like interferon and more recently Ruxolitinib plus azacitidine have been assessed in clinical trials. In addition, there are conflicting reports about the outcome after allogeneic stem cell transplantation, likely because only a very small proportion of patients, around 5%, are eligible for this treatment.

Myelodysplastic/myeloproliferative neoplasm with neutrophilia (MDS/MPN-N)

MDS/MPN-N (previously atypical CML) is a disease with a poor survival and high risk of leukaemic transformation, and it is recommended that in fit patients, allogeneic stem cell transplant should be performed. About 65% of patients have a normal karyotype. The most common myeloid mutations are *SETBP1, ASXL1, NRAS, KRAS, SRSF2* and *TET2*, involving *JAK/STAT* and *MAPK* signalling pathways, and notably the classic MPN mutations *JAK2, MPL* and *CALR* are rarely found (Table 34.3).

MDS/MPN with *SF3B1* mutation and thrombocytosis (MDS/MPN-SF3B1-T)

MDS/MPN-SF3B1-T is associated with anaemia, bone marrow dyserythropoiesis, SF3B1 mutation and typically ring sideroblasts accounting for at least 15% of erythroid precursors. The term MDS/MPN with ring sideroblasts and thrombocytosis is an acceptable term to be used for cases with wild-type SF3B1 and ≥15% ring sideroblasts. Platelet count is ≥450 × 10^9/L and megakaryocytes are large and atypical. The karyotype is usually normal. JAK2V617F is present in around 50% of cases, whereas CALR and MPL are rare (Table 34.3).

The prognosis of MDS/MPN-SF3B1-T is similar to that of *SF3B1* mutated MDS-RS, the latter usually preceding the myeloproliferative transformation. Most patients will be scored as low risk according to IPSS-R. The risk for thrombosis is similar to that in essential thrombocythemia (ET). Treatment involves erythropoiesis-stimulating agents for anaemia, and frequently aspirin and hydroxycarbamide to reduce platelet count and reduce the risk of thrombosis. The use of cytoreductive treatment, however, is debated and may aggravate anaemia, however, if cytoreduction is needed, hydroxycarbamide is the drug of choice. Further studies are needed with regard to specific treatment for MDS/MPN-RS-T. Symptoms of thrombocythemia like erythromelalgia, headache and vasomotor symptoms, and arterial as well as venous thrombosis, occur at similar frequencies as in ET.

Myelodysplasia of childhood

Childhood MDS is uncommon, around 1–4 per million children and represent 5% of haematological malignancies in children. JMML will be described separately below and myeloid proliferation associated with Down syndrome is grouped with Down syndrome-related myeloid leukaemia. The WHO 2022 recognises childhood MDS as a distinct category, separated into low and high-blast groups. Around 30% of childhood MDS cases are associated with inherited bone marrow failure syndromes or conditions that predispose to myeloid disease or germline aberrations predisposing to MDS or AML with *GATA2*,

ETV5, SRP72, SAMD9 and *SAMD9-L*, and *RUNX1* and *ANKRD26* (Table 34.2). From a genetic perspective, childhood MDS is quite different from adult MDS as many of the common myeloid mutations found in adult MDS and AML are rarely found in children, whereas RAS pathway mutations and SETBP1, ASXL1 and RUNX1 are common in both adult and childhood MDS. Monosomy 7 or del(7q) are found in 20–30% of childhood MDS and is an absolute indication for urgent allogeneic stem cell transplantation.

Juvenile myelomonocytic leukaemia

Juvenile myelomonocytic leukaemia (JMML) is a clonal disorder characterised by proliferation of granulocytic and monocytic lineages. JMML accounts for 40% of childhood MDS with an incidence of around 1.3 per million and a median onset of 2 years of age. Two-thirds of cases are boys. Most patients present with constitutional symptoms or infection and have a marked hepatosplenomegaly. Enlarged lymph nodes and tonsils as well as skin infiltration are common, and in the blood leucocytosis with neutrophilia, monocytosis and presence of myeloid precursors may be seen. The bone marrow is hypercellular with slight dysplastic features and myeloblasts frequently less than 5%. The most common chromosomal aberration is monosomy 7, seen in 25% of patients. JMML is characterised by hyperactivation of the RAS signal transduction pathway with molecular alterations in *PTPN11, NRAS, KRAS, NF1* or *CBL* genes (Table 34.3, Figure 34.2). In JMML, RAS pathway mutations are heterozygous and somatic, while JMML in neurofibromatosis type 1 (NF1) and JMML in children with CBL syndrome are characterised by germline RAS disease followed by acquired biallelic inactivation of the gene (Table 34.2). It should be noted that RAS pathway mutations are common in Noonan syndrome, and that myeloproliferation resembling JMML may occur very early in life however is frequently benign and resolves spontaneously before age 1 year. Adverse prognostic markers in JMML are high HbF synthesis, older age and thrombocytopenia. Approximately 60% of JMML patients who undergo allogeneic stem cell transplant achieve a long-term remission.

Diagnostic workup

European recommendations for patient management including diagnostic and prognostic work-up and treatment recommendation are well described at the MDS-Europe website, endorsed by the European Haematology Association.

Patient history and differential diagnoses

There are several non-clonal causes of cytopenia, bone marrow hypoplasia and marrow dysplasia to consider before establishing a diagnosis of MDS. Family history includes haematological disorders, cancer at early age, familial liver and lung disorders and special signs of genetic predisposition traits: early grey hair, excessive warts, nail dystrophy and short stature. Case history should cover previous and current chemical and drug exposure, marked weight loss, thyroid or other autoimmune diseases, recent infections and antibiotic treatment. Even the presence of ring sideroblasts is not a definitive sign of clonal disease, as they can be caused by non-clonal causes including lead intoxication, exposure to pyridoxine, or copper deficiency, excess zinc that decreases copper levels, or isoniazid, chloramphenicol or linezolid therapy. Physical examination may reveal splenomegaly and enlarged lymph nodes, or skin lesions indicative of Sweets syndrome, and vasculitis. Blood tests include vitamin B12, folic acid, and iron status, serum electrophoresis and ANA screen, as well as screening for HIV and hepatitis. In case of microcytic anaemia, the rare variant of acquired HbH disease (associated with *ATRX* mutation) should be considered. Flow cytometry of blood may exclude paroxysmal nocturnal haemoglobinuria (PNH) and the presence of large granular lymphocytic leukaemia (LGL) including T-cell receptor clonality. In case of isolated anaemia, assessment of parvovirous B19 in bone marrow could be considered. Clonal cytopenia of uncertain significance, CCUS, is presently considered a separate condition but is likely to be included under the umbrella of MDS in the future. Table 34.4 shows a comprehensive list of differential diagnoses.

Examination of blood and bone marrow cells

Examination of blood cells

Macrocytic anaemia is a predominant finding in MDS and may occur alone or in combination with either neutropenia or thrombocytopenia or both. Importantly, the haemoglobin value may be within the normal range, but significantly lower than normal for the specific individual. Some patients develop macrocytosis years before the onset of anaemia and aberrant red cell morphology or increased ferritin may be early indicators of ineffective erythropoiesis and MDS.

The red cells show anisocytosis and poikilocytosis and tend to be macrocytic and oval-shaped (Figure 34.4). Basophilic stippling (presence of many basophilic granulae in the erythrocyte cytoplasm) may also be seen. Occasionally, circulating and dysplastic erythroblasts are observed in the peripheral blood. Neutropenia is common and neutrophils often exhibit reduced granulation due to a defective formation of secondary granules. Complete agranulaity is highly specific for MDS. Pseudo-Pelger–Huët neutrophils are also frequent (these are neutrophils with dense clumping of the chromatin, and hypolobulated or non-lobulated) nucleus. Monocytosis is by definition present in CMML and monocytes are often morphologically abnormal (Figure 34.4). Monocytosis may also appear in MDS over time, especially in TET2 mutated cases, without suspicion of progression to CMML.

Table 34.4 Differential diagnoses to MDS and MDS/MPN.

Differential diagnosis	Diagnostic tests
Aplastic anaemia, pure red cell aplasia	Histology, cytology, parvovirus B19
Metastatic carcinoma	Histology, immunohistochemistry
Toxic bone marrow injury (alcohol, lead, zinc, copper deficiency, drugs)	History, lab tests See separate list
Reactive bone marrow changes (infections e.g. sepsis, HIV, hepatitis, tuberculosis and other chronic infections, autoimmune diseases, thyroid disease)	Cytology, history, lab tests
Paroxysmal nocturnal haemoglobinuria (PNH)	Immunophenotyping
Immune thrombocytopenia	History, disease course
Megaloblastic anaemia	Vitamin B12/folic acid concentration
Hypersplenic syndromes	History/clinical features (splenomegaly)
Acute leukaemia (especially erythroleukaemia, FAB-M6)	Cytology, histology, immunophenotyping, genetic and molecular genetic testing
Myeloproliferative neoplasms	Histology, cytogenetic and molecular genetic testing
Hairy cell leukaemia, large granular lymphocytic leukaemia (LGL-leukaemia)	Cytology, immunophenotyping, molecular genetic testing (*BRAF* and *STAT3*), T cell receptor
Congenital dyserythropoietic anaemia (rare)	Molecular genetic testing
Idiopathic Cytopenia of Undetermined Significance (ICUS)	See ICUS minimal diagnostic criteria
Clonal Cytopenia of Undetermined Significance (CCUS)	See CCUS

(*Source:* Adapted from MDS Right Europe, www.mds-europe.org/right.)

While often reduced in MDS in general, basophils and eosinophils may be increased in MDS/MPN.

Circulating myeloblasts may be found in all categories of MDS but are usually indicative of MDS with increased blasts. Finally, platelet count is frequently reduced, and platelets may show dysplasia with hypogranulated and/or giant forms. In CMML, circulating monoblasts (large round/oval nuclei, open chromatin, prominent nucleoli, abundant cytoplasm, variable basophilic and some azurophilic granules) and promonocytes may be present (Figure 34.4).

Examination of bone marrow

A bone marrow examination encompassing morphology and histopathology is mandatory in the diagnostic process. The ability to diagnose MDS is critically dependent on optimal staining of the marrow slides. There is often considerable interobserver variation, particularly where the dysplastic changes are subtle, but better consistency amongst observers in identifying cells, such as ring sideroblasts, pseudo-Pelger–Huët cells, micromegakaryocytes and when myeloblasts are clearly increased.

MDS bone marrow is hypercellular in the majority of patients, due to ineffective but hyperplastic erythropoiesis and granulocytopoiesis, but can be normocellular or, in 10–20% of cases, hypocellular. When assessing dysplasia, a minimum of 500 cells (with at least 100 of these non-erythroid cells) should be examined to gain an accurate and representative differential count. Dysplasia is defined by 10% or more cells of a certain lineage showing dysplastic features.

The myeloblast count is crucial for accurate disease classification, prognostication and treatment decisions and is primarily established on bone marrow smears and not on histology sections. However, when fibrosis or hypoplasia is present, myeloblast count should be assessed by immune histochemistry on biopsy sections. Myeloblasts may be agranular or granular and are distinguished from dysplastic promyelocytes, both of which may be granulated and have nucleoli, by the presence of a clearly identifiable Golgi zone in the latter (Figure 34.4).

Erythropoiesis may vary from normoblastic to megaloblastic. In MDS-RS, the erythroblasts are often poorly haemoglobinised or show cytoplasmic vacuolisation (Figure 34.4). The list of dysplastic features of erythrocytes is considerable and may include bi- or multinuclearity, bridging between nuclei, nuclear budding and fragmentation, increased pyknosis and basophilic stippling (Figure 34.4). Cytochemical iron staining should be performed in all cases of suspected MDS in order to quantify iron stores and to detect and enumerate ring sideroblasts (Figure 34.4) and pathological non-ring forms. Erythropoiesis may occasionally be severely reduced or absent. Granulopoiesis is usually hyperplastic and dysplastic including defective granulation, agranular or hypogranular cells or the presence of pseudo-Chediak–Higashi granules and nuclear hypo- or hyperlobulation (Figure 34.4). Prominent basophilic and eosinophilic differentiation and increased numbers of myeloblasts may be

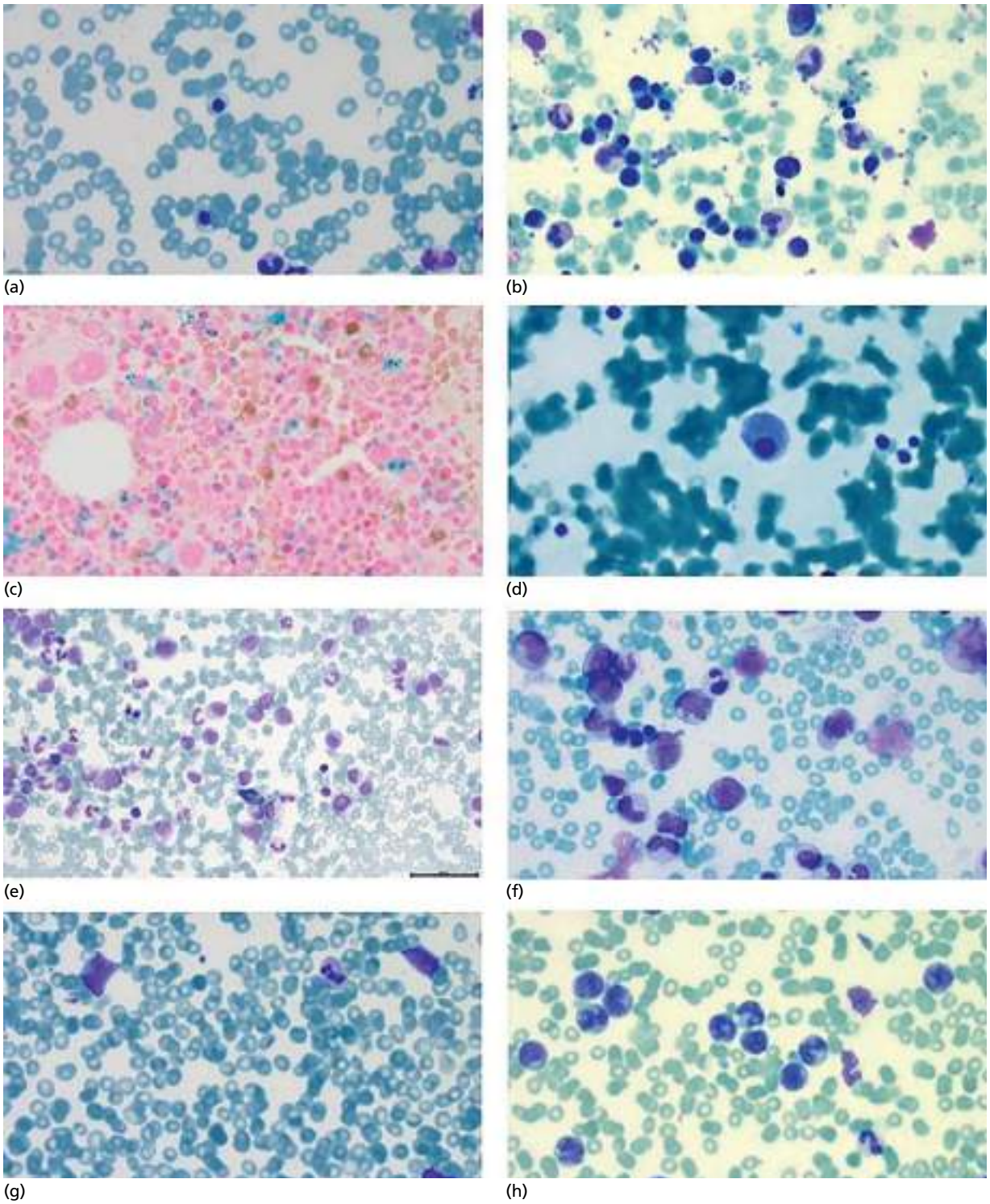

Figure 34.4 Morphology, (a) erythroid dysplasia with vacuolised erythroblasts. (b) bone marrow smears with dysplastic erythropoiesis, bilobed nuclei and irregular nuclear contours. (c) iron stain showing ring sideroblasts. (d) dysplastic monolobed megakaryocyte. (e) dysplastic neutrophils with pseudo-Pelger nuclei and hypogranulated cytoplasm. (f) myeloblasts and promonocytes in CMML, also note dysplastic neutrophils. (g) bone marrow smears from RAEB with two myeloblasts and one neutrophil granulocyte. (h) bone marrow smears with promonocytes and mature monocytes in CMML (Source: From professor Birgitta Sander, Dept of Pathology, Karolinska University Hospital and Karolinska Institutet.)

present. Megakaryopoiesis is commonly dysplastic, of which the most specific feature is the micromegakaryocyte, which is typically the size of a myeloblast with an ill-defined or blebbed outline and a single monolobulated nucleus (Figure 34.4). Other megakaryocytes may exhibit hypolobulation or contain multiple disparate nuclei due to aberrant maturation. In the 5q– syndrome, there are often increased numbers of megakaryocytes that vary in size and contain large non-lobulated nuclei.

A bone marrow trephine biopsy with histological analysis is mandatory in suspected cases of MDS, according to WHO 2022, as histology is necessary to correctly assess bone marrow architecture, cellularity, fibrosis and stromal aberrations like infiltration of metastatic solid tumours, or aberrant vascularisation. Cytological evidence of dysplasia can be found in all lineages but is most easily detected in the erythroid precursors. Abnormal clustering of megakaryocytes is often seen, as are micromegakaryocytes that can be more easily detected by immunohistochemical staining with CD61 (Figure 34.5). The finding of of abnormal localisation of immature precursors (ALIPs) may indicate an increased risk for leukaemic transformation. ALIPs are aggregates (3–5 cells) or clusters (>5 cells) of myeloblasts, usually localised in the central portion of the bone marrow away from vascular and endosteal surfaces of trabeculae.

Reticulin staining should be performed to assess fibrosis that may be mild (grade 1) or more severe, grade 2 or 3 (Figure 34.5). The presence of reticulin is often helpful in differentiating hypoplastic MDS from aplastic anaemia. In fibrotic MDS, splenomegaly is commonly absent or mild. MDS with increased blasts and fibrosis is recognised as a distinct entity in WHO 2022. In some cases, an increase in mast cells may be present, and a tryptase stain may be useful to exclude the presence of an additional systemic mastocytosis disease, especially in CMML where systemic mastocytosis may be found in around 5% of cases (Figure 34.5).

A minimal staining panel include Haematoxylin and Eosin, Giemsa, Gomori's silver stain, iron stain and immune histochemistry for CD34, p53, CD61 and glycophorin A (GPA) (Figure 34.5). In addition, staining for epithelial tumour markers may be used to exclude cytopenia caused by metastatic carcinoma. Other malignant differential diagnoses to MDS encompass MPN, acute leukaemia, aplastic anaemia, LGL, hairy cell leukaemia and congenital dyserythropoietic anaemia.

Flow cytometry

Flow cytometry immune phenotyping is recommended but not mandatory and can confirm dysplasia in peripheral blood or fresh bone marrow within 2 days of sampling. The myeloid compartment should be assessed with regard to increase of aberrant progenitors, asynchrony of myeloid differentiation antigens, decreased side scatter of granulocytes, aberrant lineage marker expression on immature or maturing cells, and in the erythroid compartment altered CD36 and or CD71 expression on nucleated cells. For CMML, immune phenotyping in peripheral blood may be useful in discriminating reactive

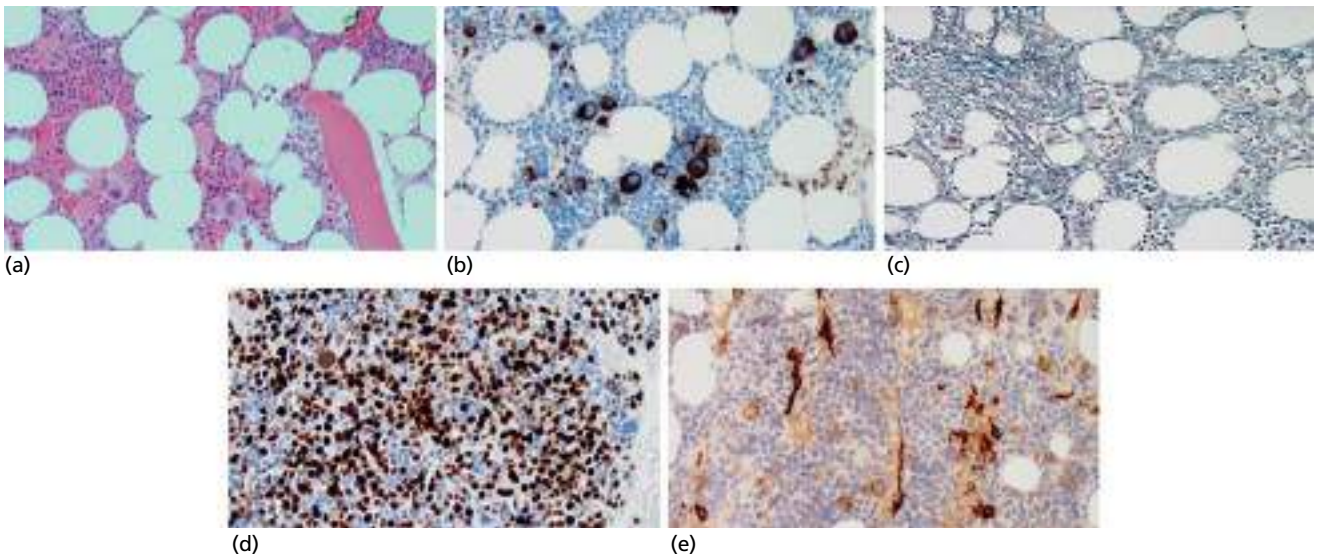

Figure 34.5 Bone marrow biopsies (a) haematoxylin-eosin stained bone marrow biopsy section showing dysplastic megakaryocytes with monolobated nuclei. (b) CD61 stain for megakaryocytes showing an increase in megakaryocyte numbers and dysplasia with several cells displaying monolobated nuclei. (c) Gomori reticular staining demonstrating an increase in reticular fibres, consistent with fibrosis grade II. (d) p53 stain with dark nuclei indicating *TP53* mutation. (e) CD117 stain showing intensely stained mature mast cells and weakly positive haematopoietic progenitor cells (Source: From professor Birgitta Sander, Dept of Pathology, Karolinska University Hospital and Karolinska Institutet.)

monocytosis from CMML, with the percentage of classical monocytes (CD14+/CD16-) being increased in CMML.

Haemoglobin electrophoresis may be performed to detect HbH that is elevated in the rare form of acquired HbH disease, and HbF that is elevated and an adverse prognostic marker in JMML.

Autoantibodies may be assessed and are frequently present in CMML, in up to 50% of cases. Serum electrophoresis is part of routine diagnostic workup to assess inflammation and presence of paraprotein.

Cytogenetic abnormalities

Cytogenetic analysis is mandatory in the diagnostic workup and remains a cornerstone in all prognostic scores. In general, at least 20 metaphases should be assessed. Clonal cytogenetic abnormalities occur in about 50% of patients with MDS and 20–50% of MDS/MPN. The most common cytogenetic abnormality in MDS is del(5q) occurring in around 15% of cases of MDS and 3% of MDS/MPN. Del(5q) isolated or in combination with one other abnormality excluding chromosome 7, and blast count <5% is called 5q- syndrome (described above). Trisomy 8, monosomy 7 or del(7q), del(20q) and -Y are the most frequent cytogenetic aberrations both in MDS and MDS/MPN. In therapy-related MDS, aberrancies of chromosome 7 are found in up to 50% of cases.

Detection of some cytogenetic abnormalities are presumptive of MDS if accompanied by cytopenia, even in the absence of morphological dysplasia. These include unbalanced aberrations such as monosomy 7 (−7), del 7q), −5, del(5q), isochromosome 17q i(17q), t(17p), 13, or del(13q), del(11q), del(12p), t(12p), del(9q), idic(X)(q13) and the more rare balanced translocations t(11;16)(q23;p13.3), t(3;21)(q26.2;q22.1), t(1;3)(p36.3;q21.2), t(2;11)(p21;q23), inv(3)(q21q26.2) and t(6;9)(p23;q34).

By contrast, the presence of trisomy 8, −Y or del(20q) require morphological evidence of dysplasia to establish the MDS or MDS/MPN diagnosis. Complex karyotype with three or more aberrancies is highly associated to adverse prognosis, as are chromosome 7 aberrancies ((−7), de(l7q)), and trisomy 8 for MDS/MPN, whereas −Y is consistent with a favourable prognosis. Tables 34.5 and 34.6 describe details on prognostic impact of chromosomal aberrations in IPSS-R and CPSS-Mol from cytogenetic aberrancies in MDS and MDS/MPN.

Molecular abnormalities

Understanding the patterns of somatic heterozygous mutations in myeloid malignancies is a rapidly expanding field and will likely bring changes to diagnostic categories as well as to prognostic tools for myeloid disease. *SF3B1* and *TP53* mutations defined disease entities already in the 2022 WHO classification and molecular abnormalities are increasingly incorporated into prognostic scores.

It is well accepted that recurrent acquired somatic mutations defining clonal haematopoiesis (CHIP) are identified in a majority of MDS patients in particular in the MDS/MPN categories. The same mutations occur across myeloid malignancies (Table 34.4). Importantly, several of these mutations occur also in the healthy elderly population, and in CCUS. There are clear differences between disease types, and e.g. CMML is more mutationally homogenous than MDS.

Targeted sequencing panel is recommended to be performed at diagnosis, on bone marrow or peripheral blood. Results are useful both for providing evidence of clonality in unclear cases like separating hypoplastic MDS from aplastic anaemia, and in cases of suspected MDS in case of a normal karyotype. Moreover, it can aid in differentiating MDS from non-clonal cytopenia e.g. in inflammatory conditions. Panel sequencing can also be used to follow disease progression or remission measuring variant allele frequency of the known mutations, to provide prognostic information with regard to type of mutations present, as well as for selecting potential targeted therapy.

While targeted mutational analysis is increasingly used in the MDS workup it is likely that also whole genome sequencing

Table 34.5 IPSS-R prognostic score for MDS.

Prognostic variable	0	0.5	1	1.5	2	3	4
Cytogenetics*	Very good	—	Good	—	Intermediate	Poor	Very poor
BM blast, %	≤2	—	>2%–<5%	—	5–10%	>10%	—
Haemoglobin (g/dL)	≥10	—	8–<10	<8	—	—	—
Platelets × 10^9/L	≥100	50–<100	<50	—	—	—	—
ANC × 10^9/L	≥0.8	<0.8	—	—	—	—	—

* Cytogenetic alterations: −Y and del(11q) are very good, normal, del(5q) and double alterations including del(5a), del(12p), del(20q) are good, del(7q), trisomy 8, trisomy 19, i(17q) and any other single or double independent clone is intermediate, −7, inv(3)/t(3q)/del(3q), double including −7/del(7q) and complex with three abnormalities are poor, and complex of more than 3 abnormalities is very poor. – indicates not applicable. The IPSS-R risk groups are: Very low ≤1.5 points, Low >1.5−3 points, Intermediate >3−4.5 points, High >4.5−6 points, and Very high >6 points, according to Greenberg 2012.

Table 34.6 CPSS molecular risk score for CMML.

Genetic risk score	**0**	**1**	**2**
Cytogenetic risk	low	intermediate	high
ASXL1	unmutated	mutated	–
NRAS	unmutated	mutated	–
RUNX1	unmutated	–	mutated
SETBP1	unmutated	mutated	–

Prognostic variable	**0**	**1**	**2**	**3**
Genetic risk score (see above)	low	Intermediate-1	Intermediate-2	high
BM blast %	<5	≥5	–	–
Leukocyte count $\times 10^9$/L	<13	≥13	–	–
Red blood cell transfusion dependence	no	yes	–	–

The genetic risk is first defined by cytogenetic risk (low risk is normal karyotype and isolated-Y, high risk is trisomy 8 and complex karyotype with three or more aberrancies, and all chromosome 7 abnormalities. All other are intermediate risk), and the presence or absence of *ASXL1*, *NRAS*, *RUNX1*, and *SETBP1* mutations. The genetic risk score is then calculated as low risk (0p), intermediate-1 (1p), intermediate-2 (2p), and high risk (3 or more points). The genetic risk is then incorporated with clinical parameters to a CPSS-Mol risk of low (0p), intermediate-1 (1p), intermediate-2 (2-3p) and high risk (4 or more points).
– indicates not applicable

will develop into a fundamental cornerstone in the diagnostic procedure of myeloid malignancies in the coming years. When sequencing costs and turnaround time decrease, whole genome and whole transcriptome sequencing may with time replace most genetic analyses enabling sensitive readout of chromosomal aberrations as well as mutations, accounting also for loss of heterozygosity.

Prognostic workup

MDS is a haematological malignancy with a varying but mainly poor prognosis. The overall median survival in large population-based registries varies between 12 and 18 months, longer in more recent cohorts due to earlier diagnosis and more patients being subject to disease-modifying treatment. The poor prognosis in most cases of MDS reflects that there are few specific treatment options reflecting the fact that malignancies with origin in the HSC compartment generally are more difficult to target.

International prognostic scoring systems

The first International Prognostic Scoring System (IPSS) was based on seven previously reported patient cohorts and was published in 1997. Based on a larger patient cohort, the cytogenetic scoring system was subsequently refined, more weight was put on the degree of cytopenia, and the percentage of bone marrow blasts was subdivided further (Table 34.5). The IPSS-R divides patients into five categories with median survival times varying between 0.7 and 5.4 years. The IPSS-R has been widely validated and used, including as a basis for enrollment if clinical trials. As with all scoring systems, there are challenges, the most prominent with IPSS-R being the intermediate risk category showing a median survival of 2.7 years and with a relatively large inter-patient variation.

It is important to note that other clinical/pathological prognostic factors not included in IPSS-R can also predict outcomes. For example, numerous studies have confirmed that the presence of a regular transfusion need is associated with a significantly worse outcome both regarding survival and transformation to AML. Another well-described risk factor is bone marrow fibrosis, associated with a worse outcome irrespective of IPSS-R.

IPSS-Molecular (IPSS-M) risk assessment score

Both the type of genetic pathway that is mutated (Figure 34.1) and the total number of mutations in an individual patient have importance for the prognosis in MDS. Commercial target sequencing panels encompassing 50–100 genes will likely be sufficient for clinical diagnostics and prognostics, the larger panels also including some of the most common hereditary alterations.

The novel prognostic scoring IPSS-M includes the variables from IPSS-R, and in addition, the molecular profiles

obtained from the panel sequencing and is from 2023 a cornerstone in national and international MDS guidelines. The IPSS-M divides patients into six risk strata and improve the prognostic precision in particular for patients with <5% marrow blasts and for the IPSS-R low and intermediate categories. Another important lesson derived from IPSS-M is the detrimental risk associated with multi-hit *TP53* mutations. Risk scores for individual patients will be difficult to calculate by hand and will require computer algorithms available on official websites. As for many other diseases 'artificial intelligence' will be one tool for guiding prognostication and optimal patient management however will never replace a competent and empathic haematologist with a general understanding of the patient´s clinical presentation and specific needs.

Prognostic scores for MDS/MPN

The two largest subtypes in this category are CMML and MDS/MPN-NOS. It was earlier shown that the myeloproliferative subtype of CMML has an overall worse outcome than the myelodysplastic subtype. The well-established and widely used CMML-specific CPSS-mol score is based on cytogenetics, peripheral blood counts, blast count and includes four myeloid mutations, *ASXL1*, *SETBP1*, *NRAS* and *RUNX1* with more weight on *RUNX1* mutation (Table 34.6). CPSS-Mol identifies four clearly separated risk groups where the low-risk group has a median survival not reached in the study and an incidence of leukaemic transformation of 0%, and the high risk has an overall survival of 18 months and a 48% risk of leukaemic transformation. IPSS-M can be used for CMML patients with leucocyte count $<12 \times 10^9$/L. For MDS/MPN-NOS, there are currently no specific prognostic scores, and despite MDS/MPN-U patients scoring lower risk points in IPSS-R, the majority will have a poor outcome.

Management and treatment

Good management of MDS is based on careful diagnostic and prognostic procedures, as well as on the patient´s individual comorbidities and preferences. Risk-based therapeutic decision-making generally uses IPSS-R, but will increasingly incorporate mutational profiles and scores also. In addition to disease-specific variables, patient-related factors are essential. Age and comorbidities, naturally, influence the spectrum of available therapies and are incorporated into comorbidity and frailty scores both for MDS and blood cancer in general. Such scores are in particular essential in the decision-making for allogeneic stem cell transplantation. Hence, accounting for both disease- and patient-related factors considerably improves risk stratification and decision-making.

Therapeutic options

Molecularly-guided precision medicine, like tyrosine kinase inhibitors for CML, is still not available for MDS and the development of novel therapeutic compounds with the propensity to markedly alter patient outcome lags behind many other blood cancers. Therapeutic options for patients with MDS vary from supportive care to allogeneic stem cell transplantation (SCT) depending on disease and patient-related risk factors with SCT being the only curative option. As websites such as MDS Europe and National Comprehensive Cancer Network (NCCN) (see reference list for web addresses) and a number of National guidelines are relatively specific about indications and dosing, this will not be detailed in this chapter.

Supportive care

Supportive care is a cornerstone in the management of all patients. Population-based studies show that about 50% of newly diagnosed patients require red blood cell transfusions and that anaemia progresses in the majority of untreated patients. Severe anaemia and transfusion-need are also associated with reduced quality of life and progression-free survival. Clinical studies indicate that QoL not only improves in patients responding to pro-erythroid growth factors, but also that higher transfusion targets are associated with improved QoL. Many guidelines therefore recommend individual transfusion targets tailored according to individual symptoms and comorbidities.

Severe thrombocytopenia with platelet transfusion need becomes more frequent with the course of disease. Consensus-based guidelines agree that platelet transfusions should be governed by trigger platelet count levels during active treatment with chemotherapy and hypomethylating agents (HMA), but mainly based on bleeding symptoms in case of chronic thrombocytopenia. Eltrombopag and romiplostim are licensed for treating severe chronic immune-mediated thrombocytopenia. These compounds have not showed to improve survival in MDS but can be used to raise platelet counts to avoid bleeding symptoms in case of severe chronic thrombocytopenia. Granulocyte-CSF is not licensed for low neutrophil counts associated with MDS but is frequently used as supportive care in case of neutropenia caused by hypomethylating agent treatment, especially after recurrent infectious events.

Iron chelation

Transfusion dependence leading to iron overload can have a negative impact on patient outcome. It is well-known from studies of thalassemia patients that severe iron overload starting in childhood significantly impairs organ function and shortens the life span. However, the exact degree of iron overload influencing outcome in adult MDS patients is not known. Based on

retrospective analyses iron chelation therapy (ICT) has been suggested to improve the outcome of lower-risk MDS patients. Only one prospective randomised phase III placebo-controlled study has been performed showing improved event-free survival in the deferasirox group with events defined as death, transformation and cardiac or hepatic abnormalities. The study design prevented evaluation of the effect on overall survival. A pragmatic view adopted by most guidelines is that iron chelation is indicated in lower-risk MDS with a permanent transfusion need but with otherwise favourable outcome, and in patients eligible for allogeneic stem cell transplantation. The drugs used for iron chelation are currently deferasirox (oral), deferioxamine (intravenous via infusion pump) and deferiprone (oral).

Haemopoietic growth factors

Erythropoiesis-stimulating agents (ESAs) constitute since long standard treatment for the anaemia of lower-risk MDS. ESAs improve haemoglobin levels and thereby QoL in at least 50% of treated patients and provided overdose and supranormal haemoglobin levels are avoided, no severe adverse events or increased risk for disease progression have been reported. Importantly, a recent large prospective registry study showed that ESA treatment significantly prolongs the time to first transfusion, if started prior to a permanent transfusion dependency. This led to an important change in the European guidelines now recommending treatment at the onset of symptomatic anaemia. If a patient responding to ESA relapses with anaemia, this is usually not associated with disease progression and the biological reasons for treatment failure are poorly understood. However, the addition of low-dose Granulocyte-CSF to erythropoietin may restore the response rate to ESA, as shown by several randomised phase II trials. The synergistic effect is particularly seen in MDS-RS and related to the anti-apoptotic effects of G-CSF on mitochondria-mediated apoptosis.

Immunosuppression

Treatment with immunosuppressive agents such as antithymocyte globulin (ATG) and Cyclosporin A may improve the cytopenia in a small proportion of patients, usually in the setting of hypoplastic MDS. There are few large prospective studies, most of them with short follow-up times, and each study has used different inclusion criteria and IST regimens. In a large meta-analysis of 570 patients with a median age of 62 years, 80% of patients had IPSS scores low or intermediate-1, the complete remission and red blood cell transfusion independence rates were 12.5 and 33%, respectively, and the progression rate to AML 8.6% per patient-year. Immunosuppressive therapy has not been confidently evaluated in relation to mutational profiles. Both European and US guidelines identify a group of younger lower-risk MDS patients with hypo- or normoplastic bone marrow and normal karyotype with the exception of trisomy 8, who may respond to immunosuppressive therapy.

Lenalidomide

Lenalidomide is licensed for lower-risk MDS with deletion 5q. Initial phase II studies showed a high probability of abrogated transfusion need in these patients as well as reversal of the del(5q) abnormality. This effect was confirmed in a Phase III randomised placebo-controlled trial, demonstrating around 50% erythroid responses and a significant proportion of partial or complete cytogenetic responses. However, lenalidomide is not a curative treatment, the median response duration is around 2 years with a clear risk of molecular and disease progression in relapsing patients. About 20% of patients with lower-risk del(5q) MDS carry *TP53* mutated subclones and as *TP53* mutated cells are insensitive to lenalidomide, treatment can lead to clonal selection with subsequent disease progression. These data led to recommendation in the NCCN and MDS-Europe MDS guidelines to treat symptomatically anaemic del(5q) MDS patients who are refractory or ineligible to erythropoiesis-stimulating agents with lenalidomide and to avoid treatment in case of *TP53* mutations.

Luspatercept

MDS-RS have been shown to display increased levels of the transforming growth factor beta (TGFβ) superfamily inhibitors in erythroid cells and plasma. Luspatercept, a recombinant fusion protein, is thought to trap TGFβ ligands and reduce SMAD2 and SMAD3 signalling, and enhance erythroid maturation and has in a recent Phase III trial been shown to increase haemoglobin levels and reduce transfusion need in MDS-RS patients, refractory or ineligible for erythropoiesis stimulating agents. Hence, luspatercept was recently licensed for use in this category and subsequent studies are ongoing to delineate its role in the therapeutic arsenal.

Hypomethylating agents

Early studies performed in the 1990s indicated clinical activity with low toxicity when used in low doses for MDS patients, and subsequently two large, randomised phase III studies were conducted. The American CALGB9221 included patients with all subtypes of MDS and showed improved overall response rate and progression-free survival in the azacitidine arm. The second randomised study, AZA-001, was designed to demonstrate a possible difference in overall survival and included patients with higher risk MDS (IPSS int-2 and high) and CMML. The median overall survival for the azacitidine-treated patients was 24.5 versus 15 months for patients assigned to the control arm consisting of either supportive care, intensive chemotherapy or low-dose cytarabine. Subgroup analyses comparing azacitidine

and the control arm demonstrated a survival benefit for azacitidine compared with low-dose cytarabine and with supportive care. There was also a survival benefit for azacitidine compared with ICT (9 months), however not significant probably due to the low number of patients in this group. Azacitidine is approved in Europe for treatment of higher-risk MDS (IPSS int-2 and high) and in the US for treatment of all MDS subgroups.

There are phase-II studies indicating a clinical effect with limited toxicity also in lower-risk MDS although the clinical benefit and the risks for this population are still unclear and no studies have hitherto provided evidence for prolonged survival.

Much effort has been given to produce clinical scores predicting response and survival after start of azacitidine treatment. Neither models based on clinical data nor clinical data in combination with gene mutations have been able to predict outcome in a robust way. Recent data have shown higher response rate for patients with *TP53* mutations. The response duration for these patients is, however, short and survival is not prolonged. By contrast, patients with *DDX41* mutations often exhibit long-lasting responses.

Based on the AZA001 study, azacitidine is licensed for CMML. The response rate for CMML patients is around 40%, but it was not until recently a large retrospective study could demonstrate a beneficial role for azacitidine in CMML. Decitabine has been evaluated in two controlled studies using the standard dose 20 mg/m^2 for 5 days. Both studies demonstrated a higher response rate (around 30%) compared to the control arm consisting of supportive care, but no survival advantage. The studies are comparable in safety and efficacy to those using azacitidine albeit lacking the Phase III data demonstrated with azacitidine. An interesting study reported high response rate for *TP53*-mutated AML patients treated with a 10-day decitabine regimen instead of the standard 5-day protocol, although with short response durations.

Intensive chemotherapy

The rate of complete remission achieved with intensive chemotherapy (e.g. cytarabine + daunorubicin) is around 50%. Patients with adverse cytogenetics, in particular those with complex karyotype, respond less well than patients with normal cytogenetics. However, cure or long-term survival of MDS with intensive chemotherapy is very rare, as chemotherapy targets cycling cells and will not target the frequently resting MDS stem cells.

Since the advent of hypomethylating agents, the use of intensive chemotherapy has decreased substantially. Intensive chemotherapy can be used to induce remission prior to allogeneic stem cell transplantation in patients with a highly proliferative disease with bone marrow blasts >10% either upfront or after hypomethylating agent failure. A clinical benefit of intensive chemotherapy for patients who are not candidates for allogeneic stem cell transplant has not been demonstrated

Other chemotherapeutic agents

For proliferative MDS/MPN and CMML, hydroxcarbamide is frequently used to reduce leucocytosis. Hydroxycarbamide may be combined with HMA treatment. For MDS/MPN with inflammation, immunomodulating agents like cyclosporin A, interferon, and more recently ruxolitinib have been empirically used. There is some support from clinical studies for ruxolitinib in combination with HMA in the MDS/MPN group including CMML.

Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (SCT) is the only therapeutic modality that leads to long-term disease control and cure in MDS. MDS is the second most common indication for SCT. The risk of severe complications is high and SCT is generally limited to fit patients up to around 70–75 years of age. In historical data, long-term survival rates of between 25 and 45% have been reported with non-relapse mortality and relapse occurring in approximately a third of the patients. More recent studies indicate improved outcome with long-term survival exceeding 50%.

Optimal timing for performing allogeneic SCT is of crucial importance. Patients with lower-risk MDS have a higher cure rate with both lower non-relapse mortality as well as lower relapse rate compared to higher-risk patients. However, lower-risk patients may have a long life-expectancy without allogeneic stem cell transplantation. The recommendation is to transplant higher-risk MDS patients upfront, and lower-risk patients at signs of progression.

Chromosomal aberrations have a large impact on relapse risk. Relapse-free survival at 5 years in the five IPSS-R cytogenetic risk groups have been reported to span between 10 and 42%. Moreover, emerging data identifies patients with *TP53*- and the *RAS*-pathway mutations as having a particularly high risk of relapse. Another important factor for the risk of relapse is disease status at the time of performing SCT where patients in remission have lower risk of relapse. Cytoreductive treatment e.g. hypomethylating agent or intensive chemotherapy is usually given for patients with a more proliferative disease or increased blasts. If such treatment changes outcome after SCT is, however, still unclear. Retrospective studies have demonstrated similar outcomes for treated and untreated patients before SCT but selection bias might contribute to these results.

Patients can be transplanted using either a reduced intensity conditioning protocol associated with higher risk of relapse but lower risk of non-relapse mortality, or a myeloablative conditioning protocol with lower risk of relapse but higher risk of non-relapse mortality. Retrospective studies have shown similar long-term overall survival.

Patients that relapse after allogeneic SCT have a poor prognosis; however, donor lymphocyte infusions and hypomethylating agent treatment may reverse the relapse in a small proportion of patients.

For MDS, there are no validated minimal residual disease markers available in MDS that can be used for initiating pre-emptive treatment. Emerging data suggest that NGS and other molecular techniques could be used to guide treatment post-SCT.

Novel therapeutic options

The BCL2 antagonist venetoclax has synergistic effects with Azacitidine in AML where it has shown to prolong survival in elderly patients not fit for intensive chemotherapy, compared to Azacitidine as monotherapy. The experience in MDS is still limited, but similar to AML, venetoclax in combination with azacitidine may be effective in reducing marrow blasts. In contrast to AML however, MDS patients seem to have more haematological toxicity with deep and prolonged cytopenia. Recent data indicate that venetoclax is not effective in patients carrying TP53 or RAS pathway mutations.

IDH1 and IDH2 inhibitors have documented clinical effects on relapsed/refractory AML carrying *IDH1/2* mutations. Several prospective studies evaluating the effect in combination with standard therapy are ongoing. Data on MDS is limited but small studies indicate an effect also in MDS. Studies on the combination of IDH1/2 inhibitors with Azacitidine are ongoing. Treatment with IDH1/2 inhibitors result in improved differentiation of the haematopoiesis and can induce a differentiation syndrome.

Eprenetapopt (APR-246) has been shown to restore tumour-suppressor function in mutant p53 cells and also to induce cell death in various cancer types. A recent study evaluated the effect of e. in *TP53* mutated MDS and AML, but failed to show a significant effect on overall survival.

With the present development of classification and prognostic systems, prospective clinical trials should be guided by molecular features rather than the present WHO categories of MDS or AML, which would facilitate access to new treatment for patients.

Future directions

MDS remains one of the more complex haematological malignancies – and one of the most difficult to treat due to the stemness of the initiating tumour cell. The development over the last decade reflects a transition from purely clinical and morphological criteria for diagnosis to a system encompassing also detailed understanding of both germline and somatic genetic abnormalities. Precision diagnostics is already in use and online tools will be an essential part of future prognostic decision-making. The relative paucity of novel therapeutic breakthroughs is presently challenged by extensive research aiming to develop precision therapy for different clinical and molecular subgroups of patients. In the end, the treating haematologist will have to encompass all this new knowledge, and the patient's general condition and personal preferences into well-functioning personalised medicine.

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Webpages

www.mds-europe.org
www.nccn.org

CHAPTER 35

Myeloproliferative neoplasms

35

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Introduction

For the purposes of this chapter, the term myeloproliferative neoplasms (MPNs) will refer to clonal disorders of haemopoiesis that lead to an increase in the numbers of one or more mature myeloid cell progeny. The chronic myeloid leukaemias would fit this definition and share pathogenetic features with some of the MPNs, but have, historically (since the discovery of the Philadelphia chromosome), been studied separately from the MPNs and are described in Chapter 33. The myelodysplastic syndromes (MDS) can also, in a minority of cases, fit our working definition of MPN, in being associated with increased numbers of mature cell progeny, but dysplasia is a major feature and with the exception of overlap syndromes (MDS/MPN) these are classified separately by the WHO (Chapter 34) and recent international consensus criteria (ICC).

This chapter will focus on the classical MPNs: polycythaemia (rubra) vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF). They share clinical, morphological and molecular features and can transform, in their course, into one another. They are clonal disorders of the pluripotent haemopoietic stem cell and have, to varying degrees, the potential to transform into acute myeloid leukaemia (AML). Secondary (non-clonal) polycythaemias and thrombocytoses will also be discussed in this chapter, as they often enter the differential diagnosis of their clonal counterparts. In addition, some of the less common MPNs will be described, the clonal eosinophilic syndromes, chronic neutrophilic leukaemia (CNL) and a group of entities which are not classified as MPN by the WHO, mastocytosis.

The molecular biology of the myeloproliferative neoplasms

Mutations in *JAK2*, *CALR* and *MPL* in PV, ET and PMF

A relationship between PV, ET and PMF was originally suggested by William Dameshek in 1951, but it was not until 2005 that a molecular basis for this was identified: an acquired activating mutation in the gene for the cytoplasmic tyrosine kinase JAK2. With sensitive detection techniques, the *JAK2 V617F* mutation is detectable in over 95% of patients with PV, and 50–60% of those with ET or PMF. JAK2 acts downstream of several cell-surface receptors including those for erythropoietin and thrombopoietin (Figure 35.1). Upon binding of a ligand such as EPO to its receptor, JAK2 becomes phosphorylated and in turn phosphorylates downstream targets, most important of which are the STATs (signal transducers and activators of transcription). PI3K and MAPK pathways are also activated and JAK2 has some direct nuclear effects through chromatin modification. Valine 617 is located in the JH2 domain of JAK2,

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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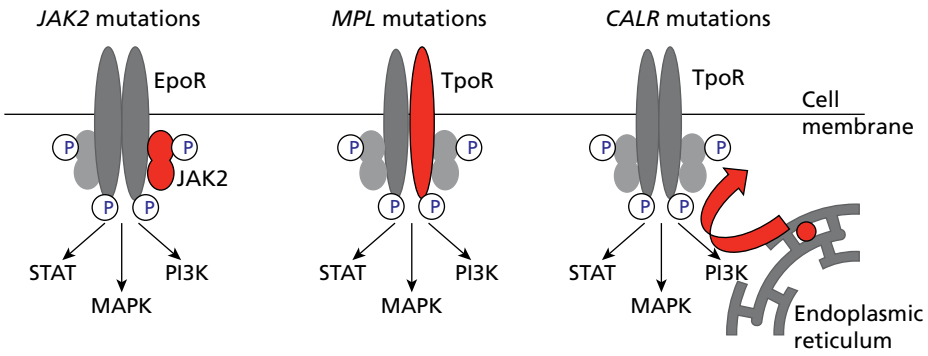


Figure 35.1 Illustration of common driver mutations in ET, PV and PMF.

which acts to repress its kinase activity. The *V617F* mutation leads to increased and constitutive kinase activity, confers cytokine independence and can cause erythrocytosis and/or thrombocytosis in mouse models.

The *JAK2 V617F* mutation is homozygous in a proportion of patients with PV and PMF, but this is uncommon in ET. The presence and size of a homozygous clone is one of the number of factors that determine how a different specific disease phenotype can arise in the context of the same pathogenic mutation. Other factors determining phenotype include any additional mutations that are acquired alongside *JAK2 V617F* and in what order they arise, as well as germline genetic background, gender and body iron stores.

Subsequent to the identification of *JAK2 V617F*, mutations in exon 12 of the *JAK2* gene were detected in almost all patients with *JAK2 V617F*-negative PV. Many different mutations have been reported, usually small in-frame insertions or deletions, but all seem to affect the pseudokinase domain, again leading to constitutive activation of the JAK2 kinase. Compared to PV patients with the *V617F* mutation, those carrying exon 12 mutations have similar rates of thrombosis, disease progression and survival. However, they tend to have a more isolated erythrocytosis, with less frequent thrombocytosis and leucocytosis, and exon 12 mutations are also not found in ET. These differences have been proposed to reflect differential interaction of the JAK2 mutant proteins with specific cytokine receptors.

Activating point mutations in the thrombopoietin receptor gene, *MPL*, were identified in 2006 and are found in 5–10% of patients with ET and PMF, but not in PV. Similar to *JAK2* V617F, these confer cytokine independence and can cause an MPN phenotype in mouse models, but *MPL*-mutant disease is characterised by thrombocytosis and/or marrow fibrosis without erythrocytosis, reflecting activation of pathways limited to the thrombopoietin receptor, compared to *JAK2*-mutant disease.

Mutations in the calreticulin (*CALR*) gene were identified in 2013 and are found in 25–35% of patients with ET and PMF, but not in PV. CALR protein is found within the endoplasmic reticulum (ER) and the frameshift mutations found in MPNs are all predicted to result in a novel C-terminal protein sequence, the commonest being a 52-bp deletion ('type 1') or 5-bp insertion ('type 2'). The two mutation types are found with similar frequencies in ET, while type 1 mutations are much commoner than type 2 mutations in PMF and in this context carry a more favourable prognosis than other mutations. Mutant CALR protein interacts with the thrombopoietin receptor (MPL) within the ER, leading to altered traffic of MPL to the cell surface and activation of downstream signalling pathways (Figure 35.1), explaining the phenotypic similarities with *MPL*-mutant disease.

Additional mutations in PV, ET and PMF

Mutations in *JAK2, CALR* and *MPL* are considered fairly specific to the classical MPN phenotype, although *JAK2 V617F*, in particular, has been reported in small numbers of patients with MDS, MDS/MPN and AML, together with a higher prevalence in MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T). In addition, mutations in other cancer driver genes are found in over half of patients with MPN. The commonest mutations are in *TET2* (10–15% of MPN patients), *ASXL1* (5–10%) and *DNMT3A* (5–10%), with a lower prevalence in splicing regulators (*SRSF2, SF3B1, U2AF1, ZRSR2*) and other regulators of chromatin structure, epigenetic functions and cellular signalling (e.g. *EZH2, IDH1, IDH2, CBL, KRAS, NRAS, STAG2, TP53*). These mutations may act through a variety of cellular mechanisms to increase the selective fitness of a haematopoietic stem cell clone harbouring a *JAK2, CALR* or *MPL* mutation, rather than driving the specific myeloproliferative phenotype. Consistent with this, these mutations are commonly found in other myeloid malignancies such as MDS and AML and some are also prevalent in age-related clonal haematopoiesis in individuals with normal blood counts (e.g. *DNMT3A, TET2, ASXL1*), although interestingly *JAK2 V617F* can also be found in the absence of blood count abnormalities in up to 3–5% of the population, depending on sensitivity of the techniques used.

These additional mutations are commoner in myelofibrosis and blast phase compared to PV and ET and some are known to carry a poorer prognostic risk (e.g. *EZH2, IDH1, IDH2, SRSF2* and *ASXL1* mutations in PMF). Biallelic *TP53* mutations are

associated with leukaemic transformation and identify a disease category with particularly unfavourable outlook.

Germline predispositions to MPNs

Although *JAK2 V617F* or exon 12, *CALR* and *MPL* mutations are acquired, several germline factors have been found to alter the risk of development of an MPN. The most important is a specific *JAK2* haplotype on chromosome 9 ('46/1'), which is present in a quarter of the population. This haplotype is enriched in patients with MPNs and *JAK2* mutations occur preferentially on the 46/1 haplotype allele. The latter observation raises the possibility that this haplotype could be more susceptible to acquisition of mutations, although an alternative explanation for the association is that mutant clones arising in patients with the haplotype have a greater proliferative advantage. Interestingly, the haplotype is associated with *JAK2* exon 12-mutant MPNs and also *MPL*-mutant disease in some studies. A number of single nucleotide polymorphisms (SNPs) in other genes (e.g. *TERT*, *TET2, SH2B3, MECOM, CHEK2, ATM, PINT, GFI1B*) have also been associated with MPNs, sometimes with varying effects between diseases; for example, a SNP between the *HBS1L* and *MYB* genes is enriched in *CALR*- and *MPL*-mutated MPNs and *JAK2*-mutated ET.

First-degree relatives of patients with MPN have a five to seven fold increased risk of developing an MPN (of any type), and familial clusters of cases are reported but account for less than 5% of cases. These families typically show incomplete penetrance, often with mixed phenotypes within a family and with the usual pattern of acquired *JAK2 V617F, CALR, MPL* and additional mutations found in sporadic cases. In most families, the predisposing allele has not been identified, but a small number of rare germline variants have been shown to contribute to such familial clusters, including structural variants at the *TCL1A/ATG2B/GSKIP* locus and *RBBP6* mutations.

Systemic mastocytosis

Mastocytosis represents an expansion of neoplastic mast cells in various tissues and is associated in the vast majority of patients with an activating mutation in the *KIT* gene, encoding the tyrosine kinase receptor for stem cell factor (SCF). The Asp816Val (*D816V*) mutation leads to ligand-independent phosphorylation of c-Kit and a consequent clonal expansion of mast cells and can be identified in the peripheral blood or bone marrow of up to 90% of patients with systemic mastocytosis (SM). A minority of patients who lack *KIT D816V* have been shown to harbour an alternative mutation either in the Asp816 codon (*D816F/H/I/Y*) or other codons (e.g. *D820G, N822K*). In other patients, the *D816V* mutation may be present but not detectable with standard assays because of a very low mast cell burden and/or suboptimal tissue sampling, and high-sensitivity assays should be available for diagnostic purposes.

Indolent and smouldering SM are primarily driven by mutated KIT, while more advanced forms of the disease (aggressive SM, SM with an associated haematological neoplasm (SM-AHN), and mast cell leukaemia) are very often associated with additional myeloid driver mutations such as in *TET2*, *SRSF2*, *ASXL1*, *RUNX1*, *JAK2*, *NRAS*, *KRAS, CBL* and *EZH2*. Additional mutations are associated with a less favourable prognosis, particularly those in *SRSF2, ASXL1* and *RUNX1*. The KIT mutation is variably detectable in the AHN cellular component, more often in chronic myelomonocytic leukaemia (CMML) than in MPN or AML.

Clonal eosinophilias

The WHO classification of tumours of haematopoietic and lymphoid tissues now includes a specific chapter for neoplasms associated with eosinophilia and recurrent rearrangements affecting genes including *PDGFRA, PDGFRB, FGFR1, FLT3* and *JAK*2. These fusions result in constitutive activation of the associated tyrosine kinase and can be associated with a variety of clinical presentations that are important to recognise because of potential implications for therapy. A *PDGFRA* rearrangement most often results from a cryptic deletion at 4q12, leading to a *FIP1L1::PDGFRA* fusion. The commonest *PDGFRB* rearrangement, t(5;12)(q31~33;p12), results in an *ETV6::PDGFRB* fusion gene but many variants have been reported and *FGFR1* rearrangements result from a variety of translocations affecting the *FGFR1* gene at 8p11. The *PCM1::JAK2* fusion currently defines an additional category in the WHO classification and other rarer fusions affecting *JAK*2 (*ETV6::JAK2, BCR::JAK2*) are also included; some cases, but not all, are associated with eosinophilia.

Eosinophilia can also occur in SM and in association with *JAK2* V617F or other rare gene rearrangements that cause tyrosine kinase activation (e.g. *ETV::ABL1*, *ETV6::FLT3*). More recently, an activating mutation in the *STAT5B* gene (*STAT5 N642H*) which was previously recognised in lymphoproliferative disorders was identified in a minority (1.6%) of patients investigated for eosinophilia. Lastly, novel recurrent acquired mutations have been reported in *JAK2* exon 13 (*Leu583-Ala586DelInsSer/Gln/Pro*) in a small number of patients with eosinophilia. These mutations also result in constitutive JAK2 activation and some patients showed a concomitant PV phenotype.

Chronic neutrophilic leukaemia

Chronic neutrophilic leukaemia (CNL) is a rare disease characterised by expansion of mature neutrophils in the blood and bone marrow granulocytic hyperplasia without dysplasia. In 2013, mutations in the gene for the colony-stimulating factor 3 receptor, *CSF3R*, were identified in patients with CNL. There are two classes of mutation with somewhat different

biochemical effects: membrane-proximal mutations, most commonly T618I, cause ligand-independent JAK-STAT signalling, while truncation mutations affecting the cytoplasmic tail cause dysregulation of SRC family and TNK2 kinases. Although *CSF3R* mutations were initially described in the MDS/MPN atypical CML, other studies have suggested that *CSF3R* mutations are fairly specific for CNL and can be detected in most patients. Other mutations reported in CNL include those in *SETBP1, ASXL1, SRSF2* and *TET2*.

Erythrocytosis/polycythaemia

Erythrocytosis

A true erythrocytosis is present when there is an absolute increase in the total body red cell volume or mass which is defined as an increase in red cell mass greater than 125% of predicted for sex and body mass. This is reflected in a raised haemoglobin (Hb) or haematocrit (Hct) although these are not entirely accurate and formal measurement of red cell mass is required to prove a true erythrocytosis is present. Without an increase in red cell mass, a reduced plasma volume may lead to an apparent erythrocytosis where there appears to be an erythrocytosis but this is only a reflection of the reduced plasma volume. The requirement for red cell mass testing was formally dropped in the recently revised WHO criteria for PV but it remains a useful test in some circumstances.

An erythrocytosis can be primary where there is an intrinsic defect in the erythroid compartment leading to increased red cell production or secondary where erythropoietin (EPO) production drives red cell production. An erythrocytosis can be congenital or have an acquired causation. Following investigation there remains a group for whom no cause for the erythrocytosis is identified termed idiopathic erythrocytosis (see Table 35.1)

Table 35.1 Causes of an erythrocytosis.

Primary (abnormality within erythroid compartment)
- Congenital
  - *EPOR* gene mutation
  - Mutations within *SH2B3*
- Acquired
  - Polycythaemia vera

Secondary (erythropoietin driven)
- Congenital
  - Mutation in genes in Oxygen sensing pathway: *PHD2*, *VHL* or *HIF2A* mutations
  - *EPO* gene gain-of-function mutation
  - Abnormal haemoglobin with increased oxygen affinity
  - Methaemoglobinaemia
  - *BPMG* mutation leading to reduced 2,3-bisphosphoglycerate
  - *PEIZO*1 mutations
  - *SLC30A10* mutations with hypermanganesemia
- Acquired
  - Hypoxia driven: high altitude, chronic lung disease, right-to-left cardiopulmonary vascular shunts (cyanotic congenital heart disease), carbon monoxide poisoning, smokers, hypoventilation syndromes including sleep apnoea
  - Local hypoxia :Renal artery stenosis, end-stage renal disease, hydronephrosis, renal cysts (polycystic kidney disease), following kidney transplantation
  - Pathological EPO production: Tumours – cerebellar haemangioma, meningioma, parathyroid carcinoma/adenomas, hepatocellular carcinoma, renal cell carcinoma, pheochromocytoma, uterine leiomyomas
  - Exogenous EPO: drug-associated erythropoietin and androgens
  - Associated with a monoclonal gammopathy (TEMPI, Clarkson's syndrome)

Idiopathic erythrocytosis (cause unknown)

Polycythaemia vera

The commonest acquired primary erythrocytosis is polycythaemia vera (PV). In this disorder, there is an acquired clone which drives an expansion in the total red cell mass, although accompanying elevations in the platelet and/or neutrophil counts are relatively common. The first description of PV was by Vaquez in 1892. In 1903, Osler published the first series of patients, identifying salient clinical features and setting PV apart from other erythrocytoses. Considerable information has been gathered about PV since the work of these pioneers, much of it due to the work of the Polycythaemia Vera Study Group (PVSG), which was set up in 1967 with the aim of optimising diagnosis and management of PV. The discovery of the V617F mutation in the pseudokinase domain of the tyrosine kinase *JAK2* in 2005 in nearly all cases of PV, was a major advance delineating the clonal nature of the disease.

Pathophysiology

PV is a stem cell disorder classically characterised by hyperplasia of all three myeloid cell lineages. The first line of evidence in support of the stem cell origin of PV came in the form of clonality studies. Using X-chromosome inactivation patterns (XCIPs) in the mid-1970s Fialkow and colleagues showed that neutrophils, erythrocytes and platelets originated from the same clone.

Erythropoiesis in PV is autonomous and does not rely on erythropoietin (EPO). Therefore, the levels of this cytokine are typically reduced in PV patients. PV progenitor cells, unlike normal ones, can survive *in vitro* and give rise to erythroid colonies (BFU-E) in the absence of added EPO (endogenous erythroid colonies, EECs). PV erythroid progenitors show an increased sensitivity to EPO and to several other growth

factors, including insulin-like growth factor-1, thrombopoietin, interleukin-3 and granulocyte/monocyte colony-stimulating factor.

When sensitive methods are used for the detection of the *JAK2* V617F mutation, over 95% of PV patients are positive. Mutations elsewhere in *JAK2* have been described in most of the V617F-negative patients who have PV by strict diagnostic criteria. These mutations cluster in exon 12 and can be of several different types, but all seem to affect the pseudokinase domain, leading to constitutive activation of the JAK2 kinase.

Diagnostic criteria

The diagnosis of PV requires the identification of features in support of this diagnosis, and the exclusion of other erythrocytoses (Table 35.1). The original diagnostic criteria were formulated by the PVSG in the 1970s. Criteria for PV have been refined over time as more is known about the pathophysiology. The current WHO criteria were published in 2022 and set Hb and Hct limits, the presence of an acquired clone and distinctive bone marrow trephine appearances. The British Society of Haematology (BSH) has reviewed the evidence and defined guidelines for making a diagnosis of PV, both are shown in Table 35.2.

Most patients can now be diagnosed on the basis of a raised haematocrit (>0.52 for men and >0.48 for women using BSH criteria) together with the presence of the *JAK2* V617F mutation. It is also important to note that the *V617F* mutation has been identified in a small proportion of other haematological malignancies (e.g. AML, chronic myelomonocytic leukaemia and MDS). However, none of these disorders is associated with a raised red cell mass and clinical distinction from PV is rarely an issue.

The role of bone marrow biopsy in the evaluation of PV remains controversial. It is required for WHO diagnostic criteria but this is predicated on the issue that a bone marrow biopsy can reliably distinguish PV from other MPNs such as ET. The WHO does suggest that 'bone marrow biopsy may not be required in patients with sustained absolute erythrocytosis if major criterion 3 and minor criterion are present but initial myelofibrosis can only be detected by bone marrow biopsy'. On that basis, a bone marrow biopsy may be useful as a baseline investigation for later comparison in younger patients with the disorder, or if there are atypical clinical and laboratory features at presentation. Hb and Hct limits in the current WHO criteria are lower and within the normal range. These are based on

Table 35.2 Diagnostic criteria for polycythaemia vera.

WHO criteria (2022)	BSH criteria
Major criteria	*JAK2-positive polycythaemia vera (requires both criteria)*
1 Elevated haemoglobin concentration (>165 g/L in men;160 g/L in women)or elevated haematocrit (>49% in men; 48% in women)	**1** High haematocrit (>0.52 in men, >0.48 in women) OR raised red cell mass (>25% above predicted)
2 Bone marrow biopsy showing age-adjusted hypercellularity with trilineage growth (panmyelosis) including prominent erythroid, granulocytic and megakaryocytic proliferation with pleomorphic, mature megakaryocytes	**2** Mutation in *JAK2*
3 Presence of *JAK2*V617F or *JAK2* exon 12 mutation	
Minor criterion	
Subnormal serum erythropoietin level	*JAK2-negative polycythaemia vera (requires A1-A4 plus another A or two B* criteria)
The diagnosis on PV requires either all three major or the first two major plus the minor criterion (*JAK2* negative PV)	**A1** Raised red cell mass (>25% above predicted) OR haematocrit ≥0.60 in men, ≥0.56 in women
	A2 Absence of mutation in *JAK2*
	A3 No cause of secondary erythrocytosis
	A4 Bone marrow histology consistent with PV
	A5 Palpable splenomegaly
	A6 Presence of an acquired genetic abnormality (excluding *BCR::ABL1*) in the haematopoietic cells
	B1 Thrombocytosis (platelet count $>450\times10^9$/L)
	B2 Neutrophil leucocytosis (neutrophil count $>10\times10^9$/L in non-smokers, $\geq12.5\times10^9$/L in smokers)
	B3 Radiological evidence of splenomegaly
	B4 Low serum erythropoietin

defined levels to separate PV, ET and iron-deficient PV rather than reflecting an actual erythrocytosis.

A few patients with clinical PV are negative for the V617F mutation. Molecular testing for *JAK2* exon 12 mutations should be undertaken in this group as above. Rarely, patients are negative for both V617F and exon 12 *JAK2* mutations. In these patients, the diagnosis of PV can be made if the other criteria set out in Table 35.2 are met (WHO first 2 major and the minor criteria). However, this group of truly *JAK2*-negative PV is very rare and repeat testing for *JAK*2 V617F on a bone marrow sample, and/or every few years on a blood sample should also be considered in these patients.

Epidemiology

Combined annual incidence of PV is reported to be around 0.84 per 100,000 of the population, with a slight male predominance. The median age at onset is 55–60 years and although incidence increases with age, PV can occur at any age, rarely, in childhood.

Clinical features

Symptoms

Many patients are asymptomatic at presentation; however, a range of symptoms are associated with PV either at presentation or later in the course of the disease. General symptoms such as tiredness are described. Sluggish cerebral blood flow secondary to the increased haematocrit is thought to underlie features such as headaches, drowsiness, insomnia, amnesia, tinnitus, vertigo, chorea and even depression. Transient visual disturbances also occur.

Pruritus occurs in about one-quarter of PV patients and in some, it may be severe and even associated with suicidal ideation. It is characteristically aquagenic, precipitated by warm baths and can be associated with erythema, swelling or even pain. Pruritus is often relieved by controlling the haematocrit, but its aetiology remains elusive. Basophilia, hyperhistaminaemia and iron deficiency may be factors.

Signs

Many skin changes are seen in PV. Plethora (Figure 35.2), dilated conjunctival vessels and rosacea-like facial skin changes are not uncommon at presentation. Brown discolouration of the skin, erythromelalgia and, rarely, Sweet's syndrome may be seen.

Hypertension is probably more common in patients with PV, as is hyperuricaemia, with gout seen in about 5% of cases. Palpable splenomegaly is seen in 30–50% of cases of PV. It is unclear if its presence affects prognosis, but it may be associated with an increased risk of progression to myelofibrosis.

Complications

Thromboembolic events

Untreated PV patients run a greatly increased risk of thrombosis, which can be arterial, venous or microvascular. The increased haematocrit leads to an increased blood viscosity, rheological abnormalities and abnormal platelet–endothelial contact. Additionally, procoagulant changes in platelets (e.g. decreased response to prostaglandin D_2), thrombocytosis and pre-existing vascular disease can all be involved in increased thrombotic risk.

Figure 35.2 Typical appearance in a patient with PV.

Arterial occlusions can lead to myocardial infarcts, strokes, transient ischaemic attacks, amaurosis, scotomata and mesenteric and limb ischaemia (Figure 35.3). Less commonly, microvascular occlusions affecting the extremities and erythromelalgia can occur. In the venous circulation, unusual sites, such as the splanchnic vessels can be involved. As a result, mesenteric, splenic and hepatoportal thromboses are recognised as presenting features of PV. This propensity to venous thrombosis in atypical sites is particularly strongly correlated with the presence of the *JAK2* V617F mutation, and indeed patients presenting with otherwise unexplained splanchnic vein thrombosis (SVT) will often be found to have the mutation, even in the absence of an overt MPN. Superficial thrombophlebitis, conventional deep venous thromboses and pulmonary emboli are also seen. Much morbidity and mortality in PV is due to thrombotic events. Arterial thrombosis is more common than venous thromboembolism (VTE). Acute thrombotic events should be managed according to current guidelines with low molecular weight heparin (LMWH) the first choice of treatment followed by vitamin K antagonists. Direct oral anticoagulants are increasingly used even though there is little data for their use in MPNs. Introduction or optimisation of cytoreductive therapy is required to maximise blood count control.

Primary and secondary prevention should include screening for and control of cardiovascular risk factors. In unprovoked VTE, indefinite anticoagulation is recommended for secondary prevention as the underlying clonal disease remains present.

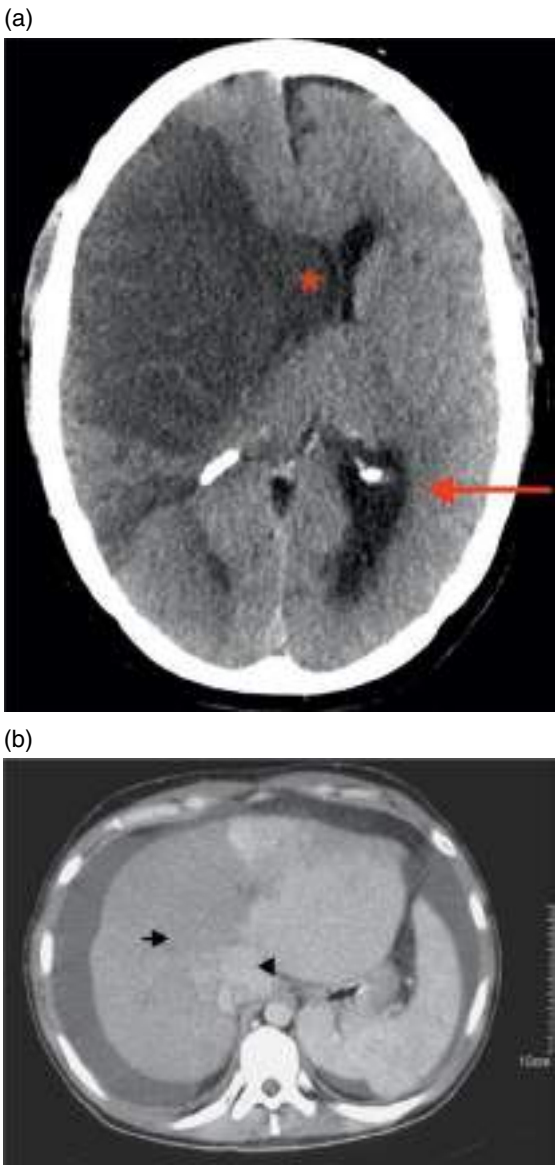


Figure 35.3 (a) Acute cerebral infarction. A 57-year-old male presented 5 days earlier with acute onset of left-sided weakness. Unenhanced CT demonstrates a large acute right middle cerebral territory infarction involving the right frontal and parietal lobes, insula and basal ganglia (asterisk — head of the right caudate nucleus). There is swelling of the infarcted brain tissue with loss of grey-white differentiation. Associated mass effect has resulted in midline shift and acute hydrocephalus of the left lateral ventricle (evidenced by dilatation of the left occipital horn, arrow (Source: Courtesy of Dr Francis Scott). (b) Polycythaemia vera presenting with the Budd–Chiari syndrome in a 28-year-old female; contrast-enhanced computerised tomography showing reduced enhancement of the right lobe of the liver (arrow) with characteristic sparing of the caudate lobe (arrowhead). On this occasion, the left lobe was also relatively spared. Marked ascites and a bulky spleen are also seen (Source: Courtesy of Dr Edmund Godfrey).

There seems to be a predilection for thrombosis in certain sites in MPN. Splanchnic vein thrombosis (SVT) involves thrombosis of the hepatic, portal, superior mesenteric and splenic veins singly or in combination. Hepatic vein thrombosis (Budd Chiari Syndrome) is commoner than portal-mesenteric axis venous thrombosis in PV and the incidence of SVT during the course of PV varies from 0.8 to 7.8%. SVT is commoner in young females. The *JAK*2 V617F mutation seems to confer a particular susceptibility. LMWH in the acute phase should be instituted unless contraindicated, with thrombolysis and stenting interventions to recanalise vessels collaborating with interventional radiology, hepatology and other colleagues. Recurrence rates are high and long-term anticoagulation is required. Cytoreduction to control blood counts with interferon is usually the first-line agent in young people. Adoption of therapeutic targets lower than the normal range may be a useful therapeutic strategy.

Cerebral vein thrombosis occurs in about 1% of MPN patients and a third of patients suffer recurrence despite anticoagulation and cytoreduction. Recurrence often involves SVT. These patients should also be treated with long-term anticoagulation and cytoreduction.

Myelofibrosis

Progression to myelofibrosis, post-PV myelofibrosis (PPV-MF) occurs in around 10–20% of PV cases 15 years from diagnosis. This figure is approximate, not least because different studies have used distinct criteria to define myelofibrotic transformation. Transformation often occurs gradually over many years, is thought to reflect accumulation of additional mutations and is associated with an increased risk of conversion to leukaemia. The management of these patients is similar to that of PMF.

Leukaemia transformation

This is the most catastrophic complication of PV, but the risk of developing acute leukaemia in PV patients treated only with venesection is thought to be small in the short term (1–3%). This risk, however, increases dramatically (more than 10-fold) when radioactive phosphorus (^{32}P), chlorambucil or irradiation are used as treatment. The median time interval between first starting such therapy and developing acute leukaemia is 5–8 years. Interestingly, in about one-third of patients transforming to acute leukaemia from a preceding *JAK2*-positive MPN, the leukaemia is *JAK2*-negative. Recent long-term studies suggest that the risk of myelodysplasia (MDS) or AML in PV patients treated with hydroxycarbamide, is approximately 20% at 20 years, although it is not clear whether this represents an effect of the drug or part of the natural history of the disease.

Prognosis and risk stratification

In the first half of the twentieth century, entirely untreated PV had a dismal prognosis, with a 50% survival of less than 2 years,

deaths being due to thromboembolic events. However, adequately treated PV now has a longer natural history with a life expectancy of over 11 years, which, bearing in mind that the average age of onset is 60–65 years represents a modest reduction in actual life expectancy. Factors predictive of poorer prognosis and increased complication rates in PV include *JAK2* mutation burden and white cell count at diagnosis, both factors probably serving as surrogate markers of disease activity.

On the basis of the biological factors, patients are classified as high or low risk for thromboembolic complications. The BSH defines high risk as age ≥65 years and/or prior PV-associated thrombotic history and this is the group that will primarily be considered for cytoreductive therapy.

Treatment

Treatment in PV is aimed at reduction of the thrombotic and haemorrhagic risk. Minimisation of the symptomatology and complications and minimisation of the risk of transformation to myelofibrosis and acute leukaemia are other aims to be considered.

The mainstay of treatment for low-risk PV is regular venesection in patients who can tolerate it. However, some 'low risk' patients may be considered higher risk in the presence of cardiovascular risk factors, elevated white blood count, extreme thrombocytosis or haematocrit uncontrolled by venesection and this group may need to be considered for cytoreductive therapy. A target haematocrit of ≤0.45 is used, following the demonstration that in patients with PV, higher haematocrit targets are associated with a significantly increased risk of thrombosis. This has been confirmed in a randomised controlled trial, CYTO-PV. Venesection has little impact on the haematocrit in the short term: the purpose of regular venesection is to induce iron deficiency, such that the haematocrit remains chronically below the target threshold of 0.45. Thus, typical venesection regimens start with phlebotomy every 2–3 weeks until the haematocrit is controlled, and thereafter phlebotomy is generally needed every 1–3 months, depending upon factors such as dietary iron intake and erythropoietic activity. Low-dose aspirin (75–100 mg daily) reduces thrombotic complications in PV and should be used in all PV patients without contraindications to this drug. Its use is supported by a randomised study (ECLAP).

High-risk patients are recommended for cytoreductive therapy. It is also recommended for patients unable to undergo venesection and in some low-risk patients with marked thrombocytosis, leucocytosis or intractable symptoms. The first-line management option of the high-risk PV patient is either hydroxycarbamide or a pegylated interferon, depending on patient and physician choice. The second line option is usually the drug not chosen for first-line use. Ruxolitinib is a second-line option for those intolerant or resistant to hydroxycarbamide. Third-line options are busulphan, ^{32}P, pipobroman or anagrelide in those where control of the platelet count is difficult.

Hydroxycarbamide (hydroxyurea) is the most commonly used drug; it is orally bioavailable and generally very well tolerated, and it will reduce both the haematocrit, leucocyte count and platelet count. The commonest complications are leucopenia or thrombocytopenia, which are dose-dependent and can usually be avoided by close monitoring of the blood count when the drug is first introduced. In susceptible patients, it can cause photosensitivity, painful leg ulcers and gastrointestinal side effects. Other important cutaneous toxicities of hydroxycarbamide include increased risk of sun-induced squamous skin lesions, these may be both cancerous and precancerous, and pigmentation can also occur. Patients should be warned about these effects and counselled about sun exposure. Hydroxycarbamide is also genotoxic and as a radiomimetic, it should be stopped during radiotherapy. The usual dose is 0.5–2 g daily.

It has been suggested that hydroxycarbamide may increase the inherent leukaemogenic risk associated with PV. This concern is largely based on studies involving small numbers of patients or patients who have also required other cytotoxic agents (and may therefore represent a subgroup with more aggressive disease). At present, there is no convincing data to show that hydroxycarbamide, when used as a single agent, significantly increases the risk of leukaemia, although a small effect cannot be excluded. The only available comparator studies have shown that the comparator treatments (e.g. pipobroman) are more leukaemogenic. Studies of hydroxycarbamide in sickle cell disease are reassuring, with long follow-up. However, sickle cell disease is not an acquired clonal disease with an inherent tendency to leukaemic transformation, so while this data is reassuring it is not directly transferable to MPN patient populations.

Interferons (IFN) are effective in controlling both the platelet/leucocyte count and the haematocrit and may induce impressive reductions in the burden of V617F-positive cells in the blood. Pegylated forms are now in widespread use with pegylated IFN-α-2a (PEG-α-2a) showing complete responses in high percentages of patients and some complete molecular remissions. Peg-α-2b has also been shown to be effective in controlling disease and proline-PEG-α-2b (Ropeginterferon) has demonstrated haematological and molecular responses in the PROUD-PV studies. This drug is now licensed for the treatment of PV. Pegylated IFNs are more costly and have to be administered by the subcutaneous route. There is a significant associated side-effect profile (including fatigue, flu-like symptoms, depression and autoimmune phenomena). They are, however, non-cytotoxic and are therefore an option in younger patients, those who are reluctant to take other cytotoxic agents and in pregnancy. Doses vary depending on the product used.

Ruxolitinib has been shown to be effective in the treatment of PV and has been approved for PV patients resistant or intolerant to hydroxycarbamide. It is very effective in the treatment of side effects such as itch in some patients. The UK MAJIC study showed that ruxolitinib was superior to other available therapies in this patient group with improved blood count control,

event-free survival and molecular responses in ruxolitinib-treated patients. This study also demonstrated a strong correlation between molecular response (50% reduction) in *JAK2 V617F* mutant allele fraction and better overall, progression-free and thrombosis-free survival.

Busulfan is sometimes used in elderly patients or if other treatments are not tolerated. It is convenient as it need only be administered intermittently but may increase the risk of leukaemia. The usual dosage is 25–75 mg as a single dose every 2–3 months, or 2–4 mg daily for 7–14 days repeated every 4 weeks until the target blood count is reached. ^{32}P is a similar option if available. It is administered by single injection in patients who can manage the radioactivity risk post-injection. It can be repeated every 1–2 years in recurrence. However, it does increase the long-term risk of leukaemia.

Anagrelide can be useful in controlling the platelet count and can be combined with hydroxycarbamide allowing lower doses of both agents. Approximately 10% of patients are completely refractory to anagrelide. The usual dose is 1–2 mg daily, but occasional patients may require doses of up to 8 mg daily. Its side effects are mainly secondary to its inotropic and vasodilatory properties (e.g. headaches, palpitations and fluid retention) although marked anaemia may also occur. Anagrelide is currently only approved in Europe for patients with ET and as second-line therapy.

Pruritus often improves with control of the haematocrit, but paroxetine, antihistamines and aspirin (in some cases) can help. There are also reports that psoralen-UV light therapy and IFN are of use. The JAK1/2 inhibitor ruxolitinib can be particularly beneficial in intractable cases.

Other causes of erythrocytosis

All disorders with an increased red cell mass which are not due to a clonal proliferation of haemopoietic progenitors are included under this heading of erythrocytosis and are classified as in Table 35.1. A group of patients do not fall into any of these categories and are given the diagnosis of idiopathic erythrocytosis. The clinical management of many of these syndromes is not well defined.

Congenital erythrocytosis

A congenital or familial erythrocytosis can be present in young patients and/or those with a family history. A number of genetic lesions can give rise to this situation. The main primary congenital erythrocytosis is due to mutations in the *erythropoietin receptor (EPOR)*. These mutations lead usually to a C-terminal truncation of the EpoR protein which is no longer down regulated and is therefore active without the addition of EPO to the receptor.

The oxygen-sensing pathway (Figure 35.4) is responsible for the maintenance of oxygen homeostasis in the cell. A number of proteins, prolyl hydroxylases (PHD), von Hippel–Lindau (VHL) and hypoxia-inducible factors (HIF) are involved in the pathway.

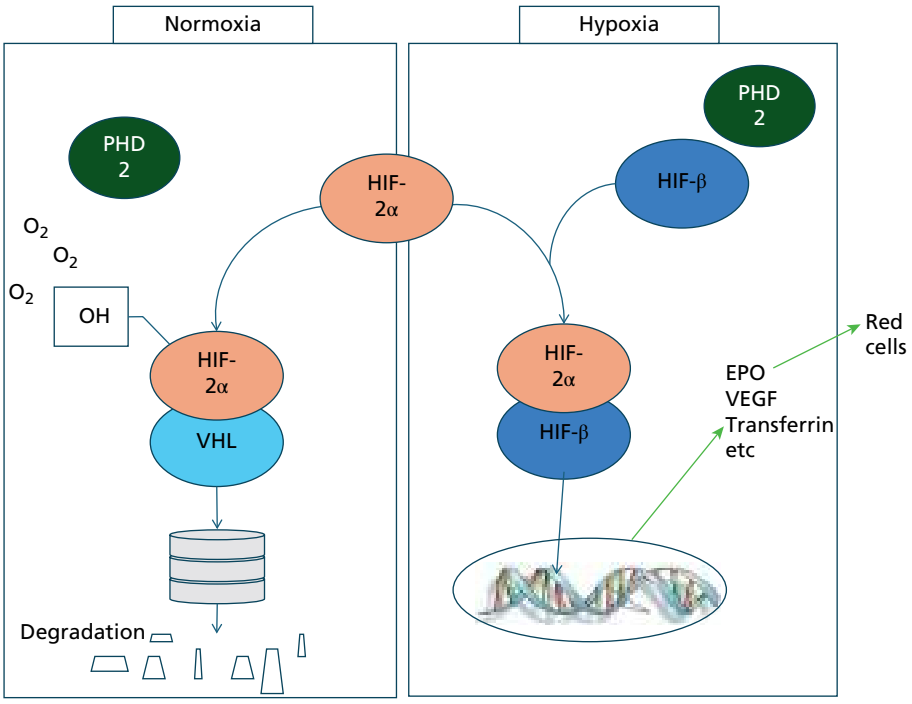


Figure 35.4 Schematic representation of the oxygen sensing pathway. EPO, Erythropoietin; HIF, Hypoxia-inducible factors; O_2, oxygen; OH, hydroxyl; PHD, Prolyl hydroxylase; VEGF, Vascular endothelial growth factor; VHL, von Hippel–Lindau.

PHD proteins have an absolute requirement for oxygen and in normal oxygen conditions hydroxylate HIF-α. Following this, VHL associates with HIF-β and is degraded in the proteasome. However, in hypoxic conditions, this does not occur. Instead, HIF-α accumulates and associates with HIF-β. A stable HIF complex is formed which translocates to the nucleus binding to promoters and enhancers of a series of genes. This leads to transcription of genes and protein production including production of EPO. Mutations in the genes in the oxygen-sensing pathway could lead to abnormal protein production which is not degraded in states of normal oxygen tension but remains as if in a state of hypoxia. Mutations of *VHL*, *PHD2* and *HIF2A* have been described causing secondary erythrocytosis.

The first mutation found in the oxygen sensing pathway was in *VHL* and was linked to erythrocytosis in the Chuvashia in the upper Volga region of Russia where there were a large number of cases. All affected cases had a homozygous mutation C598T. This has been described in other populations and compound heterozygote VHL mutations have also been seen. Autosomal dominant pedigrees with mutations in the *PHD2* and *HIF-2A* genes have been described with erythrocytosis.

There are a number of other inherited conditions which can lead to a secondary erythrocytosis: (i) An extended kindred with a mutation in the *EPO* gene itself which leads to increased translation and production of EPO has been discovered. (ii) High-affinity haemoglobins release less oxygen for a given oxygen partial pressure and may thus give rise to tissue hypoxia. This leads to an EPO-driven erythrocytosis. The pathognomonic anomaly is a left shift in the oxygen dissociation curve. There are over 90 haemoglobin variants with an increased affinity for oxygen, all dominantly inherited. Most are due to mutations in β-globin, with a small number due to mutations in α-globin. (iii) Abnormalities in red cell metabolism that lead to a reduction in intraerythrocytic 2, 3-BPG. The best characterised defect is a mutant 2, 3-BPG mutation. (iv) Hereditary methaemoglobinaemias can be due to haemoglobin mutations involving amino acids around the haem pocket (haemoglobin M disease), or secondary to enzymatic deficiencies that interfere with the generation of NADH, which is required for day-to-day methaemoglobin reduction (namely NADH reductase and cytochrome *b*5 reductase). With the chronic nature of the methaemoglobinaemia in these disorders, secondary erythrocytosis can develop. (v) Another gene recently associated with erythrocytosis is the *PIEZO1* (Piezo type mechanosensitive ion channel component 1) gene encoding for the protein, a mechanosensitive ion channel, which translates a mechanical stimulus into calcium influx. Gain-of-function mutations have been described in hereditary xerocytosis and in some cases, erythrocytosis is associated.

Management of congenital erythrocytosis

There is a sparse evidence base to guide management of the rare group of patients with congenital erythrocytosis. Low-dose aspirin is proven to reduce the incidence of thromboembolic events in acquired MPNs. As such, it seems logical to give this to those with congenital erythrocytosis in whom there is no contraindication to aspirin. Venesection would be the logical therapeutic intervention to reduce the Hct and by implication the viscosity. However, this may not be of benefit as the altered physiology due to the mutations may require a higher Hb for satisfactory oxygen supply to tissues. Venesection is only suggested if a decrease in Hb is associated with improvement in symptoms. Inhibitors of the JAK/STAT pathway and PHD2 and HIF2a may have a role in inhibition of specific defective proteins in the future.

Acquired secondary erythrocytosis

Erythrocytosis: hypoxia driven

Systemic hypoxia for a variety of reasons will lead to an EPO drive and an attempt at a corrective erythrocytosis. Lung disease including chronic obstructive pulmonary disease (COPD) and syndromes such as obstructive sleep apnoea and hypoventilation due to muscle weakness or paralysis, chronic carbon monoxide poisoning, smokers, high altitude and cyanotic heart disease can all lead to states of chronic systemic hypoxia and thus be associated with secondary erythrocytosis. Local hypoxia as in lesions in the kidney leading to a local oxygen deficit and therefore EPO production by the kidney can produce a secondary erythrocytosis. This includes renal artery stenosis and renal cysts, following renal transplantation and other renal disease.

Abnormal EPO production

EPO can be produced pathologically by a variety of tumours (see Table 35.1) where the abnormal EPO secreted by the tumour drives erythrocytosis. The other source of EPO is when there is an exogenous supply. This can either be by the administration of EPO itself or androgens which stimulate EPO release.

Idiopathic erythrocytosis

In a small proportion of patients with erythrocytosis, the criteria for the diagnosis of PV are not met and no other aetiology for the raised red cell mass can be identified. With the advent of increasingly sophisticated diagnostic tests and the identification of the molecular lesions in many inherited forms of erythrocytosis, idiopathic erythrocytosis is becoming a rare entity. Guidelines suggest aspirin if no contraindication and venesection in selected cases with the HCT target tailored to the thrombotic history and risk factors.

Reactive thrombocytosis

Thrombocytosis is most commonly reactive and secondary to increased levels of circulating cytokines that stimulate thrombopoiesis. Inflammatory, vasculitic and allergic disorders,

acute and chronic infections, malignancies, haemolysis, iron deficiency and blood loss can all lead to an increased platelet count (Table 35.3). Reactive thrombocytosis can sometimes be marked and, occasionally, the platelet count can be greater than 1000×10^9/L. Initial assessment of thrombocytosis should include comprehensive history and examination, iron studies and inflammatory markers. As a minimum a chest radiograph is recommended in patients aged over 40 years and other investigations should be considered according to clinical assessment.

Essential thrombocythaemia

Following exclusion of reactive causes of thrombocytosis, and in the absence of other blood count abnormalities that would suggest an alternative myeloid neoplasm such as chronic myeloid leukaemia (Table 35.3), patients with a persistent thrombocytosis greater than 450×10^9/L should be assessed for ET.

Table 35.3 Causes of thrombocytosis.

Reactive
- Iron deficiency
- Blood loss (acute or chronic)
- Hyposplenism/splenectomy
- Surgery
- Acute bacterial infection
  - Pneumonia, septicaemia, meningitis, diverticular abscess, etc.
- Chronic inflammation
  - Vasculitides
  - Inflammatory bowel disease
  - Connective tissue disorders
  - Rheumatoid arthritis
  - Chronic infections
- Non-myeloid malignancies
- Rebound thrombocytosis
  - Following treatment of immune thrombocytopenic purpura
  - Recovery from chemotherapy
- Drugs
  - Vincristine

Clonal myeloid disorders
- Myeloproliferative neoplasms
  - Essential thrombocythaemia
  - Polycythaemia vera
  - Primary myelofibrosis
  - Chronic myeloid leukaemia
- Myelodysplastic/myeloproliferative neoplasms
  - MDS/MPN with ring sideroblasts and thrombocytosis
- Myelodysplastic syndromes
  - MDS with isolated del(5q)

Diagnosis

A mutation in *JAK2* (*V617F*, 50–60%), *CALR* (25–30%) or *MPL* (~5%) can be detected in 85–90% of patients with ET and testing for these variants, which can be performed on peripheral blood, is a first-line investigation for an unexplained thrombocytosis. A bone marrow biopsy typically shows megakaryocytic expansion with increased large or giant forms showing abundant, mature cytoplasm and hyperlobulated nuclei (Figure 35.5). WHO criteria (Table 35.4) mandate the use of a bone marrow biopsy at diagnosis to confirm the features of ET and to distinguish it from prefibrotic myelofibrosis. The alternative criteria proposed by the BSH allow a diagnosis of ET to be made in the presence of an appropriate pathogenic mutation, if other myeloid malignancies can be excluded using other clinical and laboratory criteria (Table 35.4), although a bone marrow biopsy remains a recommended investigation in patients likely to have a long disease history or in the presence of atypical features (e.g. splanchnic thrombosis). In patients with uncomplicated ET, cytogenetic or comprehensive genomic profiling studies are not required since additional abnormalities do not change management recommendations.

In 10–15% of patients with ET, no mutation in *JAK2, CALR* or *MPN* is detectable. Patients lacking these mutations with a persistent, significant and unexplained thrombocytosis require a bone marrow biopsy for further assessment, including for alternative diagnoses. Confirmatory histology is mandatory to make a diagnosis of ET in these cases, although there can be substantial inter-observer variability in the interpretation of histological criteria for MPNs and in patients with mild or moderate thrombocytosis and no confirmatory molecular marker,

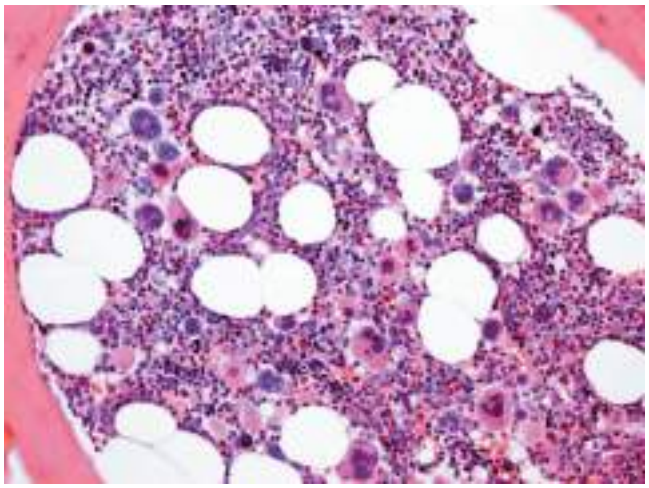


Figure 35.5 Bone marrow trephine section (haematoxylin and eosin, H&E) from a 60-year-old man with essential thrombocythaemia. Note the hypercellularity and marked increase in megakaryocyte numbers, consisting largely of clusters of mature, multilobated forms.

Table 35.4 Diagnostic criteria for essential thrombocythaemia proposed by the British Society of Haematology and World Health Organization.

BSH (2014 modification of 2010 criteria)		WHO 2022	
A1	Sustained platelet count $\geq 450 \times 10^9$/L	Major criteria	**1** Platelet count $\geq 450 \times 10^9$/L
A2	Presence of an acquired pathogenic mutation (e.g. *JAK2, CALR* or *MPL*)		**2** BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant left-shift of neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibres
A3	No other myeloid malignancy, especially PV, PMF, CML or MDS		**3** Not meeting criteria for *BCR::ABL1*$^+$ CML, PV, PMF, MDS or other myeloid neoplasms
A4	No reactive cause for thrombocytosis and normal iron stores		**4** Presence of *JAK2, CALR* or *MPL* mutation
A5	Bone marrow aspirate and trephine biopsy showing increased megakaryocyte numbers displaying a spectrum of morphology with predominant large megakaryocytes with hyperlobated nuclei and abundant cytoplasm. Reticulin is generally not increased (grades 0–2/4 or grade 0/3)	Minor criteria	Presence of a clonal marker or absence of evidence for reactive thrombocytosis
Diagnosis requires A1–A3 or A1 + A3–A5		Diagnosis requires all four major criteria or first 3 major and one minor criteria	

a confident histological diagnosis can be challenging. Although more extensive profiling of panels of myeloid genes using next-generation sequencing (NGS) can be considered, many cases of ET, particularly young patients, have no detectable recurrent genomic abnormalities when tested with such panels. The aetiology of the thrombocytosis in such patients is unclear, and it remains possible that a proportion may have a non-clonal disorder contributed to by polygenic factors. *BCR::ABL*1 analysis should be performed to exclude CML and cytogenetic studies and/or NGS panels should be considered in patients in whom appearances suggest an alternative clonal disorder such as MDS/MPN.

Epidemiology of ET

The annual incidence of ET is similar to that of PV at around 1.5–2.0 cases per 100,000 of the population. The median age at onset is 55–60 years, with a small second peak in women of reproductive age and, although it can occur at any age, it is rare in childhood.

Clinical features

Over half of patients are asymptomatic at diagnosis. Less commonly, patients may present with headaches or other microvascular symptoms such as erythromelalgia, or with vascular (thrombotic or haemorrhagic) complications. Splenomegaly is present in a minority of patients at diagnosis and is rarely more than mild. Progressive enlargement of the spleen during the course of ET should raise suspicion of evolving myelofibrosis.

In comparison to *JAK2*-negative patients, patients with *JAK2*-mutant ET show some clinical features resembling PV, including increased haemoglobin levels, neutrophil counts, bone marrow erythropoiesis and granulopoiesis, lower platelet counts, mean corpuscular volume, serum erythropoietin and ferritin. Compared to *JAK2*-mutant ET, patients with a *MPL* mutation present with a more isolated thrombocytosis and less hypercellular bone marrow, whereas those with a *CALR* mutation have lower haemoglobins and higher platelet counts.

Vascular complications and risk stratification

As with PV, thrombotic complications are the main cause of morbidity and mortality in ET. Thromboses are present in around 15–20% of patients at presentation and may be arterial or venous. The range of clinical syndromes is similar to PV, but the frequency of splanchnic thromboses is probably lower and strongly correlated with presence of the *JAK2 V617F* mutation. Bleeding is less common and less well-studied than thrombosis in ET but can be dramatic. Although alterations of platelet function have been described in MPNs, this investigation is unable to predict haemorrhagic risk. Bleeding is, however, more common in patients with a marked thrombocytosis (e.g. platelet count $>1000 \times 10^9$/L) and, in at least some cases, this is due to

Table 35.5 Revised IPSET-thrombosis risk stratification system.

Risk category	Variables
Very low	No thrombosis history + age ≤60 years + *JAK2*-unmutated
Low	No thrombosis history + age ≤60 years + *JAK2*-mutated
Intermediate	No thrombosis + age >60 years + *JAK2* unmutated
High	Thrombosis history (any age/genotype) or age >60 + *JAK2*-mutated

(*Source:* Adapted from Barbui T, Vannucchi AM, Buxhofer-Ausch et al. (2015) Practice-relevant revision of IPSET-thrombosis based on 1019 patients with WHO-defined essential thrombocythemia. *Blood Cancer J.* 5(11):e369.

an acquired von Willebrand syndrome, with a decrease in circulating high-molecular-weight multimers caused by adsorption to the surface of the excessive platelets. Testing for acquired von Willebrand syndrome is not routinely recommended but should be considered in patients with atypical bleeding or in planning major surgery in patients with an uncontrolled thrombocytosis.

A number of risk factors are associated with an increase in the risk of thrombosis in patients with ET. Patients aged over 60 years or with a previous history of thromboembolic events have consistently been shown to have an increased risk of future thrombosis and all such patients should be considered high-risk and offered cytoreductive therapy to reduce the platelet count. In addition, the *JAK2 V617F* mutation has been shown to independently increase the risk of vascular events and has now been incorporated into the revised IPSET-thrombosis score (Table 35.5) for risk stratification. Other risk factors for thrombosis reported in ET include the more standard cardiovascular risk factors (diabetes, hypertension and cigarette smoking), white cell count at diagnosis and reticulin levels at diagnosis, although these are not usually used in isolation for individual treatment decisions.

The risk of major haemorrhage in ET has also been associated with a high white cell count and more significantly with a high platelet count. This is the rationale for the recommendation that patients with a marked thrombocytosis ($>1500 \times 10^9$/L) receive treatment to reduce the platelet count, even if young.

Disease transformation

Polycythaemic transformation occurs in less than 1–2% of patients over 10 years and is seen exclusively in *JAK2*-mutant ET. Transformation to post-ET myelofibrosis is a well-recognised complication but its frequently insidious onset and the reluctance to serially study bone marrow trephine biopsies has hampered attempts to define its nature and frequency. From the available data, myelofibrotic transformation occurs in less than 10% of patients and may be commoner in *MPL*-mutant patients and those with a *CALR* type 1 mutation. The risk also increases with age and in those with higher white cell counts, lower haemoglobin and higher bone marrow reticulin grade at diagnosis. More recently, a group of 'adverse' risk mutations (*SH2B3*, *IDH2*, *SF3B1*, *U2AF1*, *EZH2*, *TP53)* have been have been described in ET, associated with less favourable myelofibrosis-free, leukaemia-free or overall survival.

Transformation to MDS or AML occurs in less than 5% of patients. In addition to adverse risk mutations, the presence of cytogenetic abnormalities and previous treatment with alkylating agents also increase this risk, although approximately a quarter of patients who develop AML have never received any cytoreduction. Approximately 3% of patients treated with hydroxycarbamide alone develop MDS or AML if followed for a median time of 8 years. As with PV, there are no data that demonstrate that hydroxycarbamide as a single agent significantly increases the risk of leukaemia, but a small effect cannot be excluded.

Treatment

Patients are stratified according to their risk of vascular complications, with those aged over 60 or with a previous history of vascular events being considered at 'high risk' of thrombosis and those with a platelet count $>1500 \times 10^9$/L also warranting cytoreductive therapy in view of haemorrhagic risk. Screening and strict management of other cardiovascular risk factors are also mandatory and use of the combined oral contraceptive pill should be discouraged.

High-risk patients

All high-risk patients are recommended to receive low-dose aspirin. This is an extrapolation of data from the ECLAP study in PV and there are no equivalent prospective data in ET. Aspirin can also improve microvascular symptoms such as erythromelalgia and headaches.

In high-risk patients, control of the platelet count with hydroxycarbamide was shown to reduce thrombotic events compared to no cytoreductive therapy in a randomised trial. The MRC high-risk PT-1 trial compared treatment with hydroxycarbamide plus low-dose aspirin to anagrelide plus low-dose aspirin. Patients receiving anagrelide plus aspirin were significantly more likely to reach the composite primary endpoint (arterial thrombosis, venous thrombosis or major haemorrhage) and more likely to discontinue their allocated treatment. Compared to hydroxycarbamide plus aspirin, treatment with anagrelide plus aspirin was associated with a significantly increased rate of arterial thrombosis, major haemorrhage and myelofibrotic transformation, but a decreased rate of VTE. These results have established hydroxycarbamide plus aspirin as first-line therapy for most high-risk patients. Anagrelide is a useful second-line agent; a subsequent smaller study (the ANAHYDRET trial) reported non-inferiority between

hydroxycarbamide and anagrelide in patients with ET, as strictly defined by the WHO-diagnostic criteria, but was underpowered to see the differences observed in the PT1 study.

Pegylated IFN-alfa can achieve good control of the platelet count in ET, although side effects may be more problematic than with hydroxycarbamide. It has not been studied as widely in ET as in PV, although the recent randomised MPDRC-112 study showed equivalence of hydroxycarbamide and pegylated IFN-alfa in PV and ET patients with increased rates of molecular remission with interferon. It is not yet known whether the molecular responses observed in some patients with ET, as in PV, will translate into reduced rates of important long-term outcomes such as thrombosis and disease transformation. IFNs remain a very useful option in the management of ET in pregnancy and are increasingly used as a first-line treatment option in younger patients.

Busulfan can achieve good control of the platelet count but is only used in patients with limited life expectancy because of concerns over its long-term leukaemogenic potential. This is true for other alkylating agents and for radioactive phosphorus (^{32}P). More recently the JAK inhibitor ruxolitinib has been assessed in patients resistant to or intolerant of hydroxycarbamide but did not achieve better rates of haematological response than alternative best available therapy. A number of agents acting through novel mechanisms including epigenetic regulation and telomere biology are currently under investigation in clinical trials.

The optimal platelet count target is not known in ET, particularly since platelet count does not correlate with risk of thrombotic events. In patients with 'high-risk' factors the aim is typically to achieve a platelet count in the normal range, although a more moderate target (e.g. $<600\times10^9$/L) may be appropriate in patients who do not have evidence of very high vascular risk and in whom first-line therapy is not fully tolerated or effective. In younger patients for whom the only indication for therapy was a marked thrombocytosis, a more relaxed platelet target can be considered.

Low-risk patients

All patients with ET were previously recommended to take low-dose aspirin. However, more recent retrospective data in low-risk disease suggested that while use of antiplatelet agents was associated with a lower rate of thrombotic events in *JAK2*-mutant patients, a higher rate of bleeding was seen in *CALR*-mutant disease. Current recommendations remain to use aspirin in *JAK2*-mutant disease, but it should be used with caution in those with a marked thrombocytosis ($>1000\,L\times10^9$/L) and is now typically omitted in young, *CALR*-mutant patients without cardiovascular risk factors.

The 'intermediate-risk' PT-1 trial randomised patients aged 40–59 years and without high-risk features to hydroxycarbamide plus aspirin or aspirin alone and found no difference in either the incidence of vascular events or the rate of progression to myelofibrosis or leukaemia (note that this was a different definition of 'intermediate risk' to the IPSET-thrombosis risk system discussed above). Routine use of cytoreduction in patients lacking high-risk factors is therefore not recommended, but may occasionally be considered for severe symptoms such as headaches, with target of symptom control rather than a particular platelet count.

Prognosis

Few studies have directly addressed survival in ET and these have reached variable conclusions about life expectancy compared to the general population. A recent multicentre study showed a median survival of 19.8 years (32.7 years if diagnosed younger than 60 years), which was worse than the age- and sex-matched population but superior to PV.

Management of pregnancy

ET is the MPN encountered most frequently in women of childbearing age, hence the most data exists for this compared to other MPN. In pregnancy, the commonest complication of ET is first-trimester miscarriage, which occurs in up to 30% of pregnancies and is thought to reflect placental microinfarcts and insufficiency. Other less frequent complications include intrauterine death, growth retardation, premature delivery and pre-eclampsia and risks of maternal thrombosis and haemorrhage are also higher than in normal pregnancy. Nonetheless, a successful outcome (live birth) is achieved in around 70% of cases with MPNs.

Management recommendations for ET in pregnancy include use of low-dose aspirin in patients who are pregnant or planning a pregnancy, in the absence of contraindications, and at minimum a period (e.g. 6 weeks) of thromboprophylaxis with LMWH post-partum when the risk of VTE is highest. Pregnancies deemed at 'high risk' (Table 35.6) should be considered for therapy with a combination of IFN, aspirin and low-molecular-weight heparin. Hydroxycarbamide and anagrelide should not be used on account of their teratogenic potential. Fetal growth and successful placentation should be closely monitored and therapies adjusted accordingly. The blood count may rise dramatically in the postpartum period. Hydroxycarbamide and anagrelide are contraindicated during breast-feeding; interferon-alfa is also excreted in breast milk but is unlikely to be absorbed intact by the baby and there are reports of successful breast-feeding while the mother was receiving IFN. Management for PV and PMF are similar, the main difference for PV being the need to ensure the Hct remains in the middle of the gestation-appropriate range (i.e. 0.45 is too high for women beyond the first trimester of pregnancy).

Uncommon causes of thrombocytosis

Aside from ET, PV and PMF, a number of other haematological malignancies can be associated with thrombocytosis. Chronic

Table 35.6 Criteria for high-risk pregnancy in myeloproliferative neoplasms (MPN).

*Sustained rise in platelet count rising to above 1500 × 10^9/L**
Previous venous or arterial thrombosis
*Previous haemorrhage attributed to MPN**
Previous pregnancy complication:
- ≥1 unexplained deaths of a morphologically normal fetus ≥10 weeks of gestation.
- ≥1 premature delivery of a morphologically normal fetus <34 weeks gestation due to:
  - **i** Severe pre-eclampsia or eclampsia defined according to standard definitions.
  - **ii** Recognised features of placental insufficiency.
- ≥3 unexplained consecutive miscarriages <10 weeks gestation, with maternal and paternal factors (anatomic, hormonal or chromosomal abnormalities) excluded.
- Otherwise unexplained intrauterine growth retardation.
- Significant antepartum or postpartum haemorrhage requiring transfusion.

Abnormal uterine artery Dopplers at 20 weeks (mean pulsatility index >1.4)

* These would represent indications for interferon only rather than interferon plus low molecular weight heparin.

myeloid leukaemia (CML) occasionally presents as an isolated thrombocytosis. MDS and MDS/MPN are generally associated with cytopenias and do not commonly present with thrombocytosis, but exceptions are MDS/MPN with ring sideroblasts and thrombocytosis and MDS with isolated del(5q), the latter being associated with thrombocytosis in one third to one-half of patients. These entities will be diagnosed on bone marrow morphology together with supplementary molecular or cytogenetic tests.

Rare cases of hereditary thrombocytosis have been described due to germline mutations in the *MPL, JAK2* and *THPO* genes. These variants are different to the germline variants which predispose to classical MPNs and account for clusters of cases within families, described above. For *MPL*, certain individual mutations (e.g. *S505N*) have been described to occur as either acquired or germline variants. *JAK2 V617F* has not been described in the germline, but a germline *JAK2 V617I* mutation was described in a family with hereditary thrombocytosis.

Prefibrotic myelofibrosis

The WHO 2016 and subsequent 2022 criteria included an entity called prefibrotic myelofibrosis (diagnostic criteria are shown in Table 35.7). The optimum management of this condition which often presents in a similar manner to ET but is said to have a worse prognosis with higher rates of thrombosis and myelofibrosis transformation has yet to be fully defined.

Table 35.7 Diagnostic criteria of prefibrotic myelofibrosis.

Prefibrotic myelofibrosis	
All three major and one minor criterion must be met	
Major criteria	Typical megakaryocyte changes accompanied by ≤grade 1 fibrosis
	Presence of *JAK2*, *CALR* or *MPL* mutations or presence of other clonal markers, or absence of evidence for reactive BM fibrosis (see Table 35.12)
	Not meeting WHO criteria for other myeloid neoplasms
Minor criteria	Anaemia not otherwise specified
	Leucocytosis ≥11 × 10^9/L
	Splenomegaly detected clinically and/or by imaging
	Increased lactate dehydrogenase
	A leucoerythroblastic blood smear

(*Source:* With permission of WHO 2022/World Health Organization).

Primary myelofibrosis

Primary myelofibrosis (PMF) is a more aggressive condition than ET, PV or pre-MF which is progressive and frequently profoundly affects quality of life for affected patients. As discussed earlier, patients with ET, PV or pre-MF are all at risk of transformation to myelofibrosis which may be known as post-ET-MF or post-PV MF. The management of patients at this stage is the same as for those with PMF with the exception of the use of prognostic scores which can perform differently in this setting. Analogous to the concept of chronic and accelerated phases in CML, it seems likely that PMF represents the presentation in accelerated phase of a previously undiagnosed MPN. Consistent with this concept, patients with PMF harbour more mutations, have more cytogenetic abnormalities and display an increased risk of leukaemic transformation

Epidemiology

The estimated annual incidence of PMF is around 0.5–1.5 per 100,000 of the population, with most patients diagnosed in the sixth decade and roughly equal involvement of the two sexes. Up to a third of patients are asymptomatic at diagnosis and many of these are discovered after unrelated blood tests show modest abnormalities, such as anaemia and thrombocytopenia. Splenomegaly is common, progressive and often massive (Figure 35.6).

Figure 35.6 Massive splenomegaly in a 53-year-old man with an 8-year history of primary myelofibrosis (PMF).

Diagnosis

A diagnosis of PMF is achieved by following established criteria which use a combination of clinical and laboratory features (Tables 35.8 and 35.9). As with all MPN, the diagnosis should be reviewed and a management plan made in the context of a multi-disciplinary format.

Laboratory features

A leucoerythroblastic blood film and the presence of red cell abnormalities in particular tear drop poikilocytes (Figure 35.7) are key diagnostic features in PMF. Mild to moderate anaemia is

Table 35.8 Diagnostic criteria for PMF.

Primary myelofibrosis (overtly fibrotic)	
All three major and one minor criterion must be met	
Major criteria	Typical megakaryocyte changes accompanied by ≥grade 2 fibrosis
	Presence of *JAK2*, *CALR* or *MPL* mutations or presence of other clonal markers, or absence of evidence for reactive BM fibrosis (see Table 35.10)
	Not meeting WHO criteria for other myeloid neoplasms
Minor criteria	Anaemia not otherwise specified
	Leucocytosis $\geq 11 \times 10^9$/L
	Splenomegaly detected clinically and/or by imaging
	Increased lactate dehydrogenase
	A leucoerythroblastic blood smear (Figure 35.7)

Table 35.9 Diagnostic criteria for post PV or post ET MF.

WHO (2022) diagnostic criteria for post PV MF	
Required criteria	Prior documentation of WHO-defined PV
	Bone marrow fibrosis ≥grade 2
Additional criteria 2 required	Anaemia or loss of need for phlebotomy/ cytoreductive treatment
	A leucoerythroblastic blood smear (Figure 35.7)
	Increasing splenomegaly
	Development of constitutional symptoms
WHO (2022) diagnostic criteria for Post ET MF	
Required criteria	Prior documentation of WHO defined ET
	Bone marrow fibrosis ≥grade 2
Additional criteria 2 required	Anaemia and a ≥20 g/L fall in haemoglobin level
	A leucoerythroblastic blood smear (Figure 35.7)
	Increasing splenomegaly
	Development of constitutional symptoms
	Increased serum lactate dehydrogenase

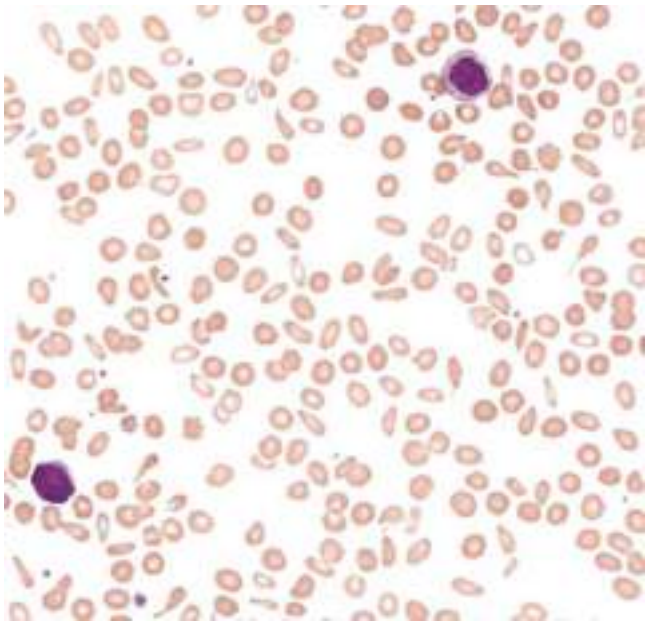


Figure 35.7 Peripheral blood film in PMF, showing a blast, an abnormal myelocyte, teardrop red cells and marked anisopoikilocytosis.

Table 35.10 Differential diagnosis of marrow fibrosis.

Haematological malignancies	*Drugs/toxins*
Primary myelofibrosis	Benzene
Chronic myeloid leukaemia	Thorotrast
Acute myelofibrosis (AML M7)	Irradiation
Myelodysplasia	*Bone disease*
Myeloma	Paget disease
Hairy-cell leukaemia	Osteopetrosis
Non-Hodgkin lymphoma	*Inflammatory diseases*
Hyperparathyroidism	Systemic sclerosis
Hodgkin disease	Systemic lupus
Hypoparathyroidism	*Other*
Systemic mastocytosis	Grey platelet syndrome
Metastatic carcinoma	TAFRO syndrome
Infections	
Tuberculosis	
Leishmaniasis	

found in most patients at presentation and worsens as myelofibrosis progresses. The anaemia is in large part due to reduced erythropoiesis but may be compounded by hypersplenism, bleeding and iron or folate deficiency. Acquired HbH disease is a rare complication. Platelet counts are raised in up to one-half of the cases at presentation and can be associated with thrombotic complications. However, progressive thrombocytopenia is a frequent occurrence and becomes increasingly troublesome as the disease progresses. Dysmegakaryopoiesis and abnormal platelet function further add to the risk of haemorrhagic complications.

Extramedullary haemopoiesis

The spleen is the commonest site of extramedullary haemopoiesis in PMF. The liver is also usually involved and this can lead to significant hepatomegaly. Unusual sites can sometimes be affected, leading to haemopoietic tumours surrounded by a capsule of connective tissue. Such sites include lymph nodes, central nervous system, skin, pericardium, peritoneum, pleura, ovaries, kidneys, adrenals, gastrointestinal tract and lungs. Many such cases remain asymptomatic, but involvement of the central nervous system can be a cause of serious morbidity. Treatment with agents such as ruxolitinib, radiotherapy or surgery, when required, almost always leads to resolution of these masses.

Systemic symptoms

A hypermetabolic state presenting with fevers, anorexia, weight loss and night sweats develops in many cases of PMF, sometimes early on in the disease. Other symptoms are often present in particular fatigue, bone pain and spleen-related symptoms such as pain and early satiety. The presence of specific symptoms – weight loss, night sweats and fever are prognostically significant. It is generally recommended that symptoms should be assessed using a specific score such as the MPN-symptom assessment form (MPN-SAF) or MPN-10 (Figure 35.8).

Bone marrow

Attempts at bone marrow aspiration often yield a dry-tap or a haemodilute sample, making aspirate morphology of limited diagnostic value. Sufficient material can often be obtained from either bone marrow or peripheral blood to assess the karyotype, which can help exclude diagnoses such as CML. Other chromosomal abnormalities may be found in up to 60% of cases, as detailed above. Abnormalities of chromosomes 5 and 7 are usually found in patients with prior exposure to genotoxic agents and are associated with a poor prognosis.

Bone marrow trephine biopsy is essential to make a diagnosis of PMF. Initial stages are characterised by an increase in bone marrow cellularity in association with a disorganisation of marrow architecture and the presence of abnormal large megakaryocytes often occurring in clusters (Figure 35.9). Bone marrow fibrosis becomes increasingly dominant and progressively replaces haemopoiesis. Intrasinusoidal haemopoiesis can sometimes be seen at this stage (Figure 35.9). The degree of fibrosis is best demonstrated using silver impregnation, which stains reticulin fibres (see Figure 35.10). Collagen fibres are best demonstrated using a trichrome stain. The degree of fibrosis can be graded from 0 to 4 (Bauermeister) or MF-0 to 3 (EU consensus) according to severity. In a minority of cases of advanced PMF, osteosclerosis ensues, with thickening of the trabecula and extensive deposition of osteoid. Such changes may be evident on plain radiography and MRI imaging.

Prognosis

Historically the median survival for patients with PMF was regarded as 3–5 years, but the range is very wide; ruxolitinib and improvements in general care have improved this in recent years. The most widely used algorithms to determine prognosis are shown in Table 35.11 and are the International Prognostic Score System (IPSS) and Dynamic (D)IPSS or DIPSS-plus. Increasingly molecular markers are having an impact upon prognosis, and have been included in newer prognostic scores such as MIPPS70, MIPSS70+ and GIPSS, Table 35.12 and for Post ET or Post PV MF the MYSEC score Table 35.13.

GIPSS, genetically-inspired prognostic scoring system, Survival quotes are for all age groups; HMR, high molecular risk mutations include *ASXL1, SRSF2, EZH2, IDH1, IDH2*

Fill out the form below to track the burden of your symptoms.

Symptom: 1 to 10, 0 if absent and 10 being worst imaginable

Please rate your fatigue (weariness, tiredness) by circling the one number that best describes your WORST level of fatigue during the past 24 hours

Fatigue										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Circle the one number that describes how much difficulty you have had with each of the following symptoms during the past week

Filling up quickly when you eat (early satiety)										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Abdominal discomfort										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Inactivity										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Problems with concentration - compared to before my diagnosis										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Night sweats										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Itching (pruritus)										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Bone pain (diffuse, not joint pain or arthritis)										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

Fever (> 37.8°C or 100°F)										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(DAILY)

Unintentional weight loss last 6 months										
0	1	2	3	4	5	6	7	8	9	10
(ABSENT)										(WORST IMAGINABLE)

To help you get a clear overall picture of how you are feeling, you can add up all your scores to calculate your Total Symptom Score. **Total:** ☐

Figure 35.8 An example of a symptom tracker for MPN patients.

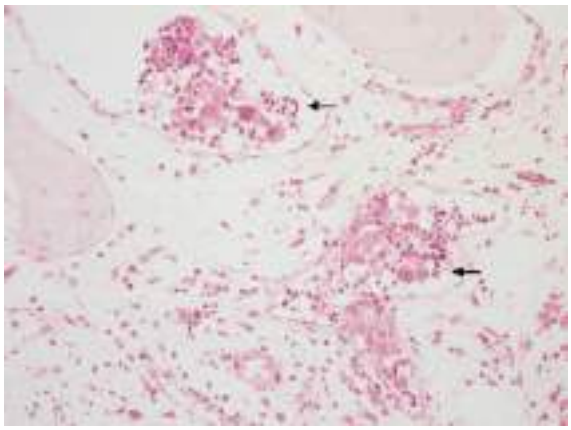
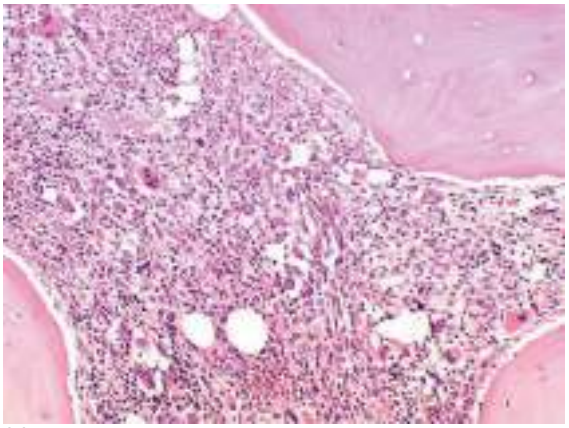
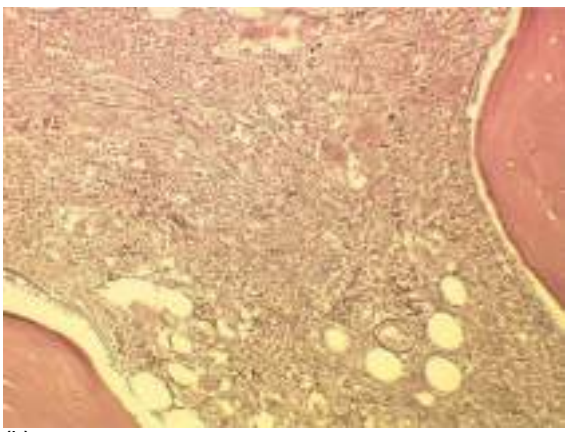


Figure 35.9 Bone marrow trephine section in PMF (H&E). Note the presence of two dilated sinusoids (arrows) containing immature haemopoietic cells, including megakaryocytes.



(a)



(b)

Figure 35.10 Bone marrow trephine sections from a patient with early-stage PMF. The H&E stain (a) shows hypercellularity, disorganised architecture, increase in megakaryocyte numbers and prominent sinusoids. The silver stain (b) also shows a marked increase in reticulin fibres.

and for GIPSS and MIPSS70+ version 2.0 *U2AF1Q157*; MIPSS70, mutation-enhanced international prognostic system for transplant-age patients (age ≤70 years); MIPSS70+ version 2.0 and karyotype enhanced international prognostic system; VHR, very high-risk karyotype (single/multiple abnormalities of −7, inv(3)/3q21, i(17q), 12p−/12p11.2, 11q−/11q23 and autosomal trisomies (e.g., +21, +19) other than +8 or +9).

Treatment

The only curative treatment for PMF is allogeneic stem cell transplantation, but this is only appropriate for a small proportion of patients who are fit enough, and have a suitable donor. In the remaining cases, therapy remains supportive and aimed at alleviating symptoms. The development of JAK inhibitors, initially ruxolitinib and the more recently approved fedratinib and momelotinib have radically altered the therapeutic algorithm for the management of PMF. Treatment is generally focused upon specific features of the disease (Table 35.14 below).

The control of symptoms and splenomegaly with JAK inhibitors such as ruxolitinib (a JAK1 and JAK2 inhibitor) and the more recently approved fedratinb (a JAK2 inhibitor) has enabled significant treatment advances for patients with PMF linked with improved quality of life and in some cases survival. These drugs, however, do not cure the disease or induce molecular remissions. Both may worsen anaemia and thrombocytopenia which may require discontinuation or the addition of other therapies. There is more real-world experience with ruxolitinib which has highlighted immunosuppressive effects leading to opportunistic infections, non-melanoma skin cancers and its positive use in inflammatory conditions such as graft versus host disease. Abrupt withdrawal of ruxolitinib is not recommended as this can lead to rapid return of splenomegaly and symptoms. There is insufficient experience with fedratinib to comment regarding immunosuppression or withdrawal but concerns with regard to Wernicke's encephalopathy have been raised with this drug and thiamine levels should be carefully monitored.

Anaemia is a common problem, and it is important to exclude another underlying cause in particular bleeding from varices due to portal hypertension. A variety of agents can be used. Human recombinant erythropoietin has shown promise in small clinical studies of anaemia in PMF, usually where endogenous Epo levels are below 250–500 IU. Momelotinib, an inhibitor of JAK2, JAK2 and ACVR1, has recently been approved and can improve anaemia in some patients. Alternatively, androgens (such as danazol), corticosteroids or immunomodulatory agents such as thalidomide may be used. Lenalidomide has been evaluated in MF but is generally recommended for the rare patients who have MF and a 5q− cytogenetic abnormality. Splenectomy may also have a residual

role for transfusion-dependent anaemia. Splenic irradiation is an alternative to splenectomy in some cases and it can significantly reduce splenic size, albeit transiently. This procedure is not without complications, as it can lead to life-threatening cytopenias. Radiotherapy can also be useful for treating pockets of extramedullary haemopoiesis involving vital organs or bodily cavities.

Thrombocytopenia is a significant challenge in the setting of PMF and options are similar to those for anaemia with the exception of ESA, splenectomy is unlikely to be successful in that setting.

Cytoreductive therapy such as hydroxycarbamide or interferon can be useful in the management of some aspects of PMF, such as leucocytosis, constitutional symptoms and troublesome thrombocytosis.

Table 35.11 Risk stratification for PMF patients.

IPSS prognostic score				
Prognostic variable	*0*	*1*		
Age in years	≤65	>65		
WBC count × 10^9/L	≤25	>25		
Haemoglobin g/L		<100		
Peripheral blast %	<1	>1		
Constitutional symptoms	No	Yes		
Risk assignment: Low = 0; Intermediate 1 = 1; Intermediate 2 = 2; High = 3+ Median survivals are 135, 95, 48 and 27 months, respectively				
DIPSS prognostic score				
	0	*1*	*2*	
Age in years	≤65	>65		
WBC count × 10^9/L	≤25	>25		
Haemoglobin g/L			<100	
Peripheral blast %	<1	>1		
Constitutional symptoms	No	Yes		
Risk assignment: Low = 0; Intermediate 1 = 1 or 2; Intermediate 2 = 3 or 4; High = 5 or 6 Median survival: not reached, 14.2, 4 and 1.5 years, respectively				
DIPSS plus prognostic score				
Points from DIPSS	*0*	*1*	*2*	*3*
DIPSS prognostic group points	Low risk 0	Intermediate 1 risk 1	Intermediate 2 risk 2	High risk 3
To the DIPSS prognostic group add one point each for:				
Platelet count × 10^9/L		<100		
Red cell transfusion required	No	Yes		
Unfavourable karyotype*	No	Yes		
Risk assignment number of points: Low = 0; Intermediate 1 = 1; Intermediate 2 = 2 or 3; High = 4 to 6 Corresponding median survival estimates: 185, 78, 35 and 16 months				

* Unfavourable karyotype includes +8, −7/7q−, i(17q), inv(3), −5/5q−, 12p−. 11q23 rearrangements and complex karyotypes.

Table 35.12 Newer prognostic models in primary myelofibrosis incorporating newer molecular and cytogenetic data.

	MIPSS70 (3 tiered)		MIPSS70+ version 2.0 (5 tiered)		GIPSS (4 tiered)
	Genetic variables	***Clinical variables***	***Genetic variables***	***Clinical variables***	***Genetic variables***
	Type 1/like CALR absent (2 points)	Haemoglobin <10 g/dL (1 point)	VHR karyotype (4 points)	Severe anaemia (2 points)	VHR karyotype (2 points)
	One HMR mutation (2 points)	Leucocytes 25 × 109/L (2 points)	Unfavourable karyotype (3 points)	Moderate anaemia (1 point)	Unfavourable karyotype (1 points)
	≥2 HMR mutations (2 points)	Circulating blasts ≥2% (1 point)	Type 1/like CALR absent (2 points)	Circulating blasts ≥2% (1 point)	Type 1/like CALR absent (1 point)
		Constitutional symptoms (1 point)	One HMR mutation (2 points)	Constitutional symptoms (2 points)	ASXL 1 mutation (1 point)
		Bone marrow fibrosis ≥grade 2 (1 point)	≥2 HMR mutations (3 points)		SRSF2 mutation (1 point)
					U2AF1Q157 mutation (1 point)
Risk group (median survival)					
Very low risk			0 points (not reached)		
Low risk	0–1 points (not reached)		1–2 points (16.4 years)		0 points (26.4 years)
Intermediate-1 risk					1 point (8 years)
Intermediate risk	2–4 points (6.3 years)		3–4 points (7.7 years)		
Intermediate-2 risk					2 points (4.2 years)
High risk			5–8 points (4.1 years)		≥3 points (2 years)
Very high risk	≥5 points (3.1 years)		≥9 points (1.8 years)		

Severe anaemia, haemoglobin <8 g/dL in women and <9 g/dL in men, Moderate anaemia, haemoglobin 8–9 g/dL in women and 9–10 g/dL in men.

Table 35.13 A specific prognostic model for myelofibrosis secondary to PV and ET-Prognostic Model (MYSEC-PM) has also been developed.

Risk factor	Points assigned	Risk	Median survival
Age	via nomogram	Low	Not reached
Haemoglobin <110 g/L	2	Intermediate 1	9.3 years
Platelets $<150 \times 10^9$/L	1	Intermediate 2	4.4 years
Circulating blasts ≥3%	2	High risk	2 years
CALR unmutated genotype	2		
Constitutional symptoms	1		

Table 35.14 Treatment options for myelofibrosis according to clinical need.

Clinical need	Treatment option
Anaemia	Transfusion
	Anabolic steroid
	Thalidomide/Pomalidomide/Lenalidomide
	Splenectomy
	Momelotinib
	Being tested: luspatercept, hepcidin antagonists, BET inhibitors, LSD1 inhibitors,, BCL2 family inhibitors
Symptomatic splenomegaly	Ruxolitinib*
	Fedratinib*
	Momelotinib*
	Hydroxycarbamide
	Interferon
	Splenectomy
	Splenic irradiation
	Being tested: wide variety of agents other JAK inihibitors, BET inhibitors, LSD1 inhibitors, BCL2 family inhibitors
Extramedullary haematopoiesis	Ruxolitinib
	? Fedratinib
	Radiotherapy
Risk of thrombosis	Antiplatelet agents
or thrombosis recurrence	Anticoagulants vitamin K or DOAC
	Ruxolitinib
	Hydroxycarbamide
	Interferon
Constitutional symptoms/	Ruxolitinib*
Quality of life	Fedratinib*
	Momelotinib*
Improved survival	Allogeneic stem cell transplant
	Ruxolitinib*
	? Fedratinib*

* Other JAK inhibitors such as pacritinib are in advanced development.

Mastocytosis

Mastocytosis comprises a heterogeneous group of rare haematological neoplasms characterised by the accumulation of clonal mast cells in the skin or extracutaneous organs. As discussed earlier more than 90% of patients with mastocytosis will harbour the *C-KIT* D816V mutation. Paediatric mast-cell disease overall has a good prognosis and will not be discussed here. Approximately 1:10,000 people worldwide are estimated to have mastocytosis. In 2017 and subsequently in the 2022 update, the World Health Organization (WHO) created a separate category for mastocytosis with three broad subtypes: cutaneous mastocytosis (CM), SM and an extremely rare variant mast cell sarcoma (see Figure 35.11 and Table 35.15 for classification of mastocytosis and diagnostic criteria). The WHO 2022 classification introduced a new disease category of bone marrow mastocytosis (absence of skin lesions and basal serum tryptase below 125 ng/mL).

The majority of patients have CM; the rarer condition SM is diagnosed when extracutaneous organs such as bone marrow, liver, spleen, lymph nodes and gastrointestinal tract are involved. SM is further subdivided into non-advanced or advanced diseases. Advanced systemic mastocytosis (AdvSM) comprises patients with aggressive SM, SM with an associated haematological neoplasm (SM-AHN, up to 70% of AdvSM) and mast cell leukaemia. In patients with SM-AHN the concomitant haematological neoplasms are usually myeloid, the most prevalent being CMML, MDS, MPNs and AML. In this group, additional mutations in *ASXL1*, *SRSF2*, *TET2*, *RUNX1* or *RAS* may be seen. The prognosis in these patients is unfavourable and is usually dictated by the prognosis of the AHN.

Investigations

Clinical features of mastocytosis can be highly suggestive of the disease, but diagnosis usually requires histological and biochemical confirmation. Routine investigations should include a full blood count, liver function tests, bone profile and a serum tryptase. Tests for histamine metabolites in 24-hour urine specimens are probably no more useful than measurements of serum tryptase. A genetic variable that may influence tryptase levels and symptom profile is Hereditary alpha Tryptasemia (HαT), an autosomal dominant trait defined by an increased copy number of the *TPSAB1* gene encoding alpha tryptase.

Bone marrow aspiration and trephine biopsy allow assessment of bone marrow involvement. Mast-cell aggregates can be visualised on conventional haematoxylin and eosin-stained sections (Figures 35.12 and 35.13). Immunochemistry using mast cell tryptase antibodies (MCT) and CD2, CD25 are important. Flow cytometry to look for expression of CD2 and CD25 in bone marrow mast cells may be useful, as this phenotype is not seen in normal mast cells. Quantitative PCR methods should be used to detect *C-KIT* mutations in bone marrow or peripheral blood as NGS is not sensitive enough to detect low disease burden. NGS for the detection of the presence of additional mutations is useful for predicting prognosis.

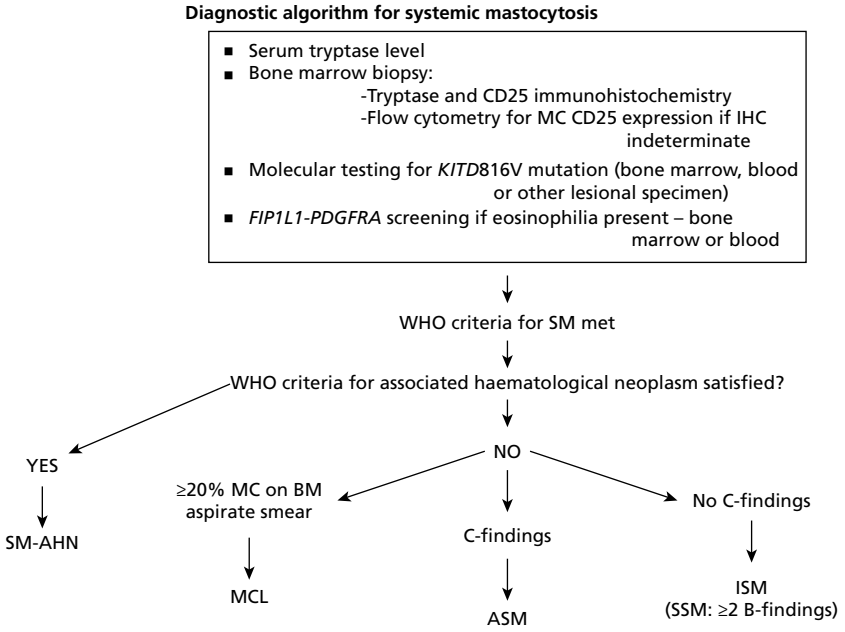


Figure 35.11 Diagnostic algorithm for systemic mastocytosis. ASM, aggressive systemic mastocytosis; IHC, immunohistochemistry; ISM, indolent systemic mastocytosis; MC, mast cell; SM, systemic mastocytosis SM-AHM MCL mast cell leukaemia; SSM, smouldering systemic mastocytosis.

Table 35.15 World Health Organization (WHO 2022) classification and criteria for diagnosis of systemic mastocytosis.

Classification

Cutaneous mastocytosis

Urticaria pigmentosa/maculopapular cutaneous mastocytosis (UP/MPCM) with monomorphic or polymorphic subtypes
Diffuse cutaneous mastocytosis (DCM)
Cutaneous mastocytoma (isolated or multilocalised)

Systemic mastocytosis

Bone marrow mastocytosis (BMM)
Indolent systemic mastocytosis (ISM)
Smouldering mastocytosis (SSM)
Aggressive systemic mastocytosis (ASM)
Systemic mastocytosis with an associated haematologic neoplasm (SM-AHN)
Mast cell leukaemia (MCL)

Mast cell sarcoma

Diagnostic criteria for systemic mastocytosis

The diagnosis of SM can be made when the major criterion and one minor criterion or at least three minor criteria are present

Major criterion

Multifocal, dense infiltrates of mast cells (≥15 mast cells in aggregates) detected in sections of bone marrow and/or other extracutaneous organs

Minor criteria

a >25% of all mast cells are atypical on bone marrow smears or are spindle-shaped in dense and diffuse mast cell infiltrates in sections of bone marrow/other extracutaneous organ(s).
b Detection of an activating point mutation in *KIT* in bone marrow, blood or other extracutaneous organ.
c Mast cells in bone marrow, blood or another extracutaneous organ(s) aberrantly express one or more of the following antigens: CD2, CD25, CD30.
d Serum total tryptase persistently exceeds 20 ng/mL (unless there is an associated myeloid neoplasm, in which case this parameter is not valid). In the case of a known Hereditary α-Tryptasemia, the tryptase level should be adjusted.

'B' findings

1 High mast cell burden shown on BM biopsy: >30% infiltration of cellularity by mast cells (focal, dense aggregates) and/or serum total tryptase level > 200 ng/mL and/or KIT D816V VAF ≥10% in bone marrow or peripheral blood leucocytes.
2 Signs of dysplasia or myeloproliferation, in non-mast cell lineage(s), but insufficient criteria for definitive diagnosis of an associated haematological neoplasm (AHN), with normal or only slightly abnormal blood counts.
3 Hepatomegaly without impairment of liver function, palpable splenomegaly without hypersplenism, and/or lymphadenopathy on palpation or imaging.

'C' findings

1 Bone marrow dysfunction caused by neoplastic mast cell infiltration, manifested by ≥1 cytopenia(s) (ANC $<1.0 \times 10^9$/L, Hb<100 g/L and/or platelet count $<100 \times 10^9$/L).
2 Palpable hepatomegaly with impairment of liver function, ascites and/or portal hypertension.
3 Skeletal involvement with large osteolytic lesions with/without pathological fractures or bone pain (pathological fractures caused by osteoporosis do not qualify as a 'C' finding.
4 Palpable splenomegaly with hypersplenism.
5 Malabsorption with hypoalbuminaemia and/or weight loss due to gastrointestinal mast cell infiltrates.

ANC, absolute neutrophil count; Hb, haemoglobin; ng, nanograms; ml, millilitre; l, litre; VAF, variant allele frequency.

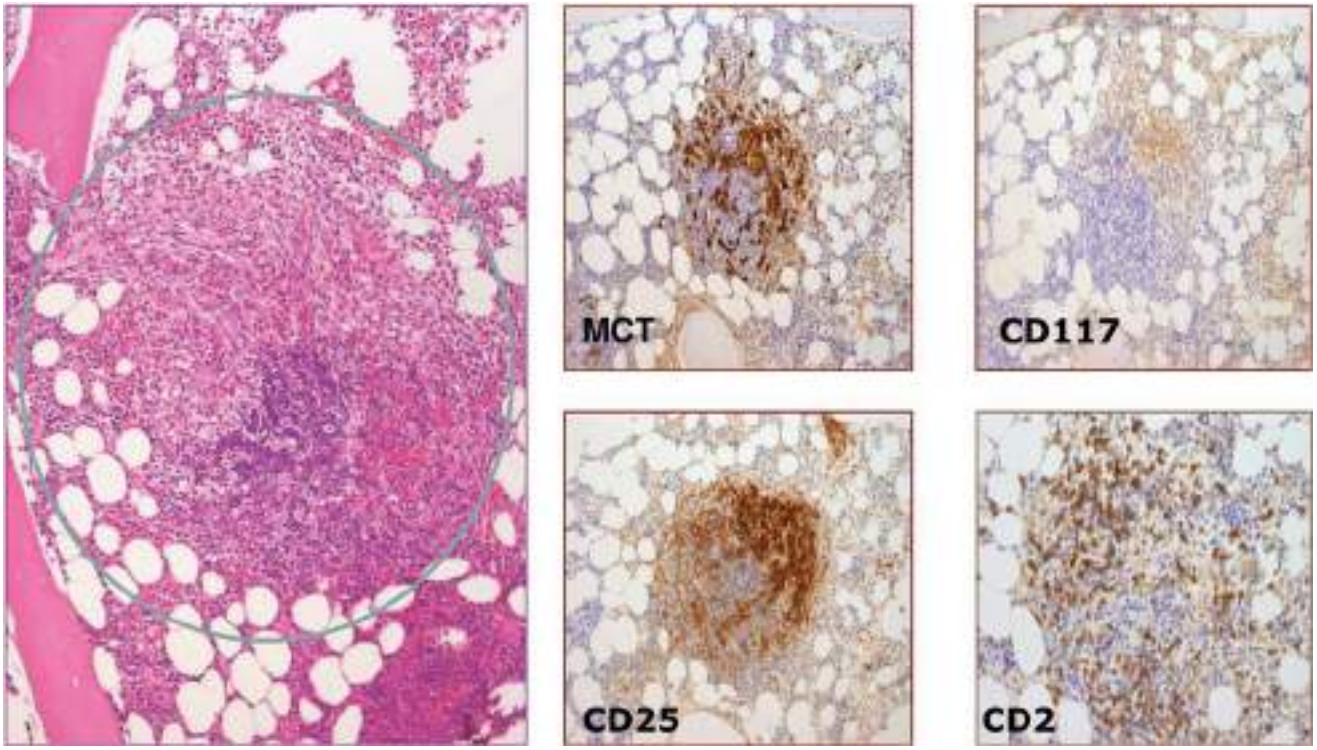


Figure 35.12 Systemic mastocytosis involving the bone marrow (H&E). Malignant whorls of rounded and spindle-shaped mast cells are seen infiltrating the bone marrow in a paratrabecular distribution (circle). Expression of CD2, CD25 and mast cell tryptase is seen (Source: Reproduced with permission from Dr Deepti Radia).

Abdominal ultrasound or computerised tomography should be performed to look for hepatosplenomegaly and lymphadenopathy. Plain radiography and bone densitometry can be used to look for bone involvement and osteoporosis. Endoscopies with biopsies or liver biopsies can be useful if gut or liver involvement is suspected.

Clinical features of systemic mastocytosis

Cutaneous manifestations

Maculopapular cutaneous mastocytosis is the usual presenting feature in children and adults with isolated mastocytosis. Yellowish–brown lesions, usually macular and sometimes papular, appear in a patchy distribution. Less commonly, there is diffuse involvement of the skin, which becomes thickened and darker brown (Figure 35.14). Pruritus is common, as is flushing, and some cases develop haemorrhagic bullous disease. Wealing of lesions upon rubbing is known as Darier's sign.

Constitutional features

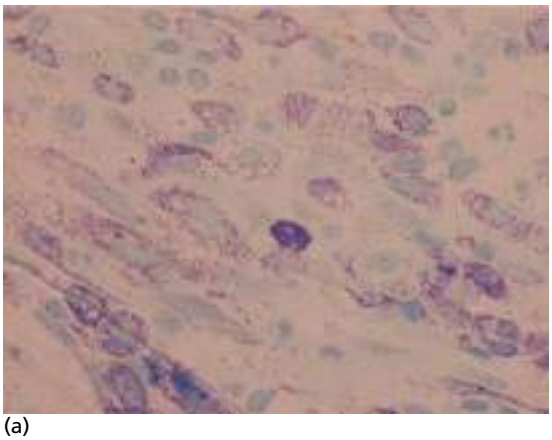
Symptoms due to mast cell mediator release vary amongst mastocytosis patients. Episodes of flushing, angioedema or even anaphylaxis, with or without any specific triggers can occur. Gastrointestinal symptoms include abdominal pain, diarrhoea, nausea, vomiting with gastritis and peptic ulceration occurring due to hyperhistaminaemia. Severe cases may develop malabsorption. Osteoporosis is well recognised and can sometimes lead to pathological fractures. Fever, fatigue and weight loss can sometimes ensue and may result from the release of cytokines such as tumour necrosis factor-α (TNF-α) and IL-1.

Systemic disease

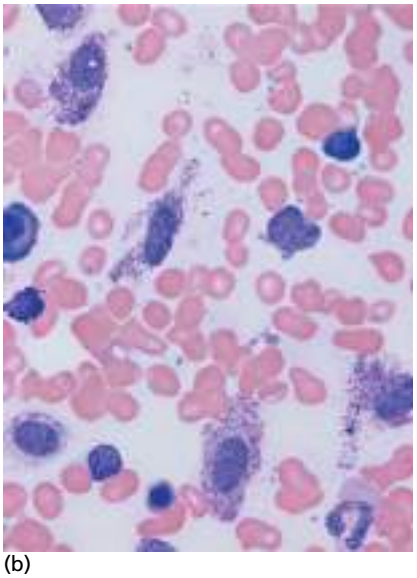
Peripheral blood cytopenias occur due to mast-cell infiltration of the bone marrow and splenomegaly. Hepatosplenomegaly is more common in cases associated with another clonal haematological disorder. Symptoms of organ failure due to infiltration are characteristic of advanced systemic mastocytosis.

Treatment

Despite significant advances in the understanding of its pathophysiology, no curative treatment exists for mastocytosis (aside from the very rare patients who are eligible for



(a)



(b)

Figure 35.13 Toluidine blue stain of bone marrow aspirate (a) and trephine biopsy section (b) in systemic mastocytosis. Abnormal spindle-shaped mast cells containing metachromatic granules are seen.

allogeneic stem cell transplant), the management of which remains symptomatic.

There are three main components to the management of mastocytosis:

1 Avoidance of factors that can trigger mediator release from mast cells.

2 Symptomatic management due to mediator release: acute and chronic.

3 Reduction of the mast cell burden/organ infiltration.

Avoidance of triggers

Avoidance of triggers of mast-cell mediator release is primarily an exercise in patient education. Severe reactions due to systemic mast-cell mediator release are difficult to predict in patients with

Figure 35.14 Female with diffuse maculopapular cutaneous mastocytosis: patient has given signed consent for photograph to be used for educational purposes.

mastocytosis and do not correlate well with disease category, mast-cell burden or severity of other symptoms. All patients and relevant healthcare workers should be warned of particular triggers, including general anaesthesia, contrast radiography and insect stings. Known mast-cell activators such as morphine and dextran should only be used with caution. Patients, regardless of whether they have had previous anaphylaxis or not, should carry injectable adrenaline and they, their family and friends should be instructed in its intramuscular administration. Local mediator release in cutaneous mastocytosis can be moderated by avoidance of triggering factors such as friction and heat.

Symptomatic management of mediator release

Acute systemic mast-cell mediator release should be treated in much the same way as other forms of anaphylaxis. Treatment with adrenaline and intravenous fluids should be started as soon as possible, with early involvement of intensive care specialists in severe cases. A short course of corticosteroids may be required. Antihistamines (H_1 and H_2 blockers) should be introduced and continued long term if the episode was particularly severe or recurrent.

Symptoms of chronic mediator release are the commonest clinical problem in mastocytosis. Symptomatic cutaneous disease should be managed with the help of a dermatologist. Treatments include H_1 and H_2 blockers, topical corticosteroids and PUVA for severe disease. Non-life-threatening systemic symptoms such as flushing, abdominal pain and diarrhoea should be treated with H_1 and H_2 blockers, mast cell stabilisers e.g. sodium cromoglycate and corticosteroids. Inhibitors of prostaglandin synthesis, such as aspirin and non-steroidal anti-inflammatory drugs, can also be useful. Aspirin should always be started with caution as it can initially lead to acute mediator release. Gastrointestinal disease usually responds to mast-cell stabilisers such as sodium chromoglycate. Leucotriene antagonists may also be useful. Peptic ulcer and reflux disease should be treated with proton pump inhibitors. Osteoporosis should be treated with bisphosphonates and may be prevented with bisphosphonates or calcium and vitamin D supplementation. Bone density should be recorded and monitored according to the severity of osteoporosis. Tricyclic anti-depressants are useful in neuropathic pain. Radiotherapy can help with severe localised pain.

Cytoreductive therapies

Patients with ISM who are still symptomatic despite maximal anti-mediator treatments and those with AdvSM should be considered for cytoreductive or targeted treatments. IFN-α, usually given in combination with oral corticosteroids, and cladribine has been found to result in partial remissions in some patients but can cause severe myelosuppression with risk of infections.

Tyrosine kinase inhibitors (TKIs) demonstrate efficacy in patients with AdvSM, imatinib in *C-KIT* negative patients or with unknown mutational status and patients with well-differentiated SM variant with *F522C* transmembrane mutation/wild type *KIT*. Midostaurin, an oral multi-kinase inhibitor of wild-type *KIT* and *D816V* mutation as well as *FLT*3, *PGRFR*α/β and2 has been approved for patients with AdvSM in 2017 by the FDA and EMA demonstrating decreases in mast cell burden, reduction in spleen size, bone marrow mast cell burden with improved symptom scores. The FDA recently approved avapritinib (June 2021), an oral-directed C-KIT D816V inhibitor for patients with AdvSM. Significant reductions were seen in mast cell burden, clearance of mast cell aggregates and reduction in C-KIT allele burden becoming undetectable in some patients with this agent.

Finally allogeneic bone marrow transplantation is an option for those patients with a suitable donor and more so with the promising results shown in the clearance of mast cells with the new TKIs. Treatment of any associated haematological disorder should be undertaken as appropriate for that disorder and the overall prognosis is usually that of the latter.

Eosinophilia

An eosinophilia can be present in a range of conditions, and for the most part, does not itself cause harm to the patient. However, eosinophilia can be a marker of underlying disease which requires treatment and rarely in the form of hypereosinophilic syndrome (HES) which will cause tissue damage. The approach to the investigation and management of this manifestation is complex and outlined below (Table 35.16).

Table 35.16 Suggested evaluation of a patient with persistent eosinophilia, following a thorough history and examination.

In all cases of eosinophilia:
- Full blood count and blood film
- Routine biochemical tests including renal and liver function tests, bone profile, lactate dehydrogenase
- Erythrocyte sedimentation rate and/or C-reactive protein
- Vitamin B_{12} assay

In patients who are otherwise well with mild to moderate eosinophilia between 0·5 and $1{\cdot}5 \times 10^9$/L, further testing may not be indicated

Patients with systemic symptoms or those with persistent eosinophilia (at least $1{\cdot}5 \times 10^9$/L), with or without suspected organ damage, should be investigated for possible secondary causes with the following:

How to test for organ damage:
Cardiac assessment:

Chest radiography, electrocardiogram, echocardiogram, serum troponin T. If there is a suspicion of cardiac involvement discuss with cardiologist see Figure 35.15

Pulmonary assessment:
Pulmonary function tests including spirometry, O_2 saturation and transfer factor of the lung for carbon monoxide (TLCO)

Thrombotic event:
An unprovoked thromboembolic event is regarded as a manifestation of tissue damage

(*Continued*)

Table 35.16 (*Continued*)

For those with a suspected clonal eosinophilia
- See algorithm in Figure 35.16 below including molecular tests and serum tryptase

In those with a suspected allergic aetiology:
- Serum total immunoglobulin E (IgE)
- Allergen-specific IgE tests
- Skin-prick testing for specific allergies

In those with a suspected non-allergic dermatological cause:
- Skin biopsy

In those with a suspected infectious cause:
- Fresh stool microscopy for ova, cysts and parasites
- Serological tests for suspected parasitic infections e.g. strongyloidiasis, schistosomiasis, filariasis, toxocariasis where appropriate
- Consider the need to test for human immunodeficiency virus (HIV) or human lymphotropic virus 1 (HTLV-1) in patients with probable opportunistic infections

In those with a suspected gastrointestinal cause:
- Upper gastrointestinal endoscopy, small bowel endoscopy and/or colonoscopy/sigmoidoscopy
- Serum amylase
- Serology for coeliac disease-related autoantibodies (anti-tissue transglutaminase)

In those with a suspected connective tissue disorder
- Antinuclear activity (ANA) or anti-double stranded DNA (dsDNA) antibodies
- Antibodies to cyclic citrullinated peptide (CCP)

In those with a suspected vasculitis:
- Antineutrophil cytoplasmic antibodies (ANCA)
- Serology for hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), cytomegalovirus (CMV) and parvovirus B19

In those with suspected respiratory disease:
- Appropriate imaging
- Bronchoscopy with bronchoalveolar lavage/endobronchial ultrasonography

In those with a suspected lymphoma, non-haematological malignancy or T-cell driven eosinophilia:
- Appropriate imaging and tissue biopsy
- Peripheral blood T-cell immunophenotyping and T-cell receptor gene rearrangement studies

Miscellaneous:
- Tests for athero-embolic disease
- Immunoglobulins and C1 esterase levels

Figure 35.15 Cardiac magnetic resonance imaging scan in a 65-year-old man with HES, showing a rim of subendocardial fibrosis (arrow).

A suggested testing algorithm for clonal eosinophilia is shown in Figure 35.16. In a non-urgent situation, it is prudent to do the least invasive tests first with peripheral blood analysis for *FIP1L1::PDGFRA* and serum tryptase where an underlying mast cell condition is considered.

Management of eosinophilia

In the absence of a clear cause with no associated end-organ damage after careful evaluation the best course is likely to be monitoring only. For eosinophilia secondary to a known cause, then treatment relates to the underlying cause. If it is necessary to treat the eosinophilia in addition to the underlying cause then high doses of corticosteroids are helpful. It is important to consider adding the anti-parasitic ivermectin. The remainder of

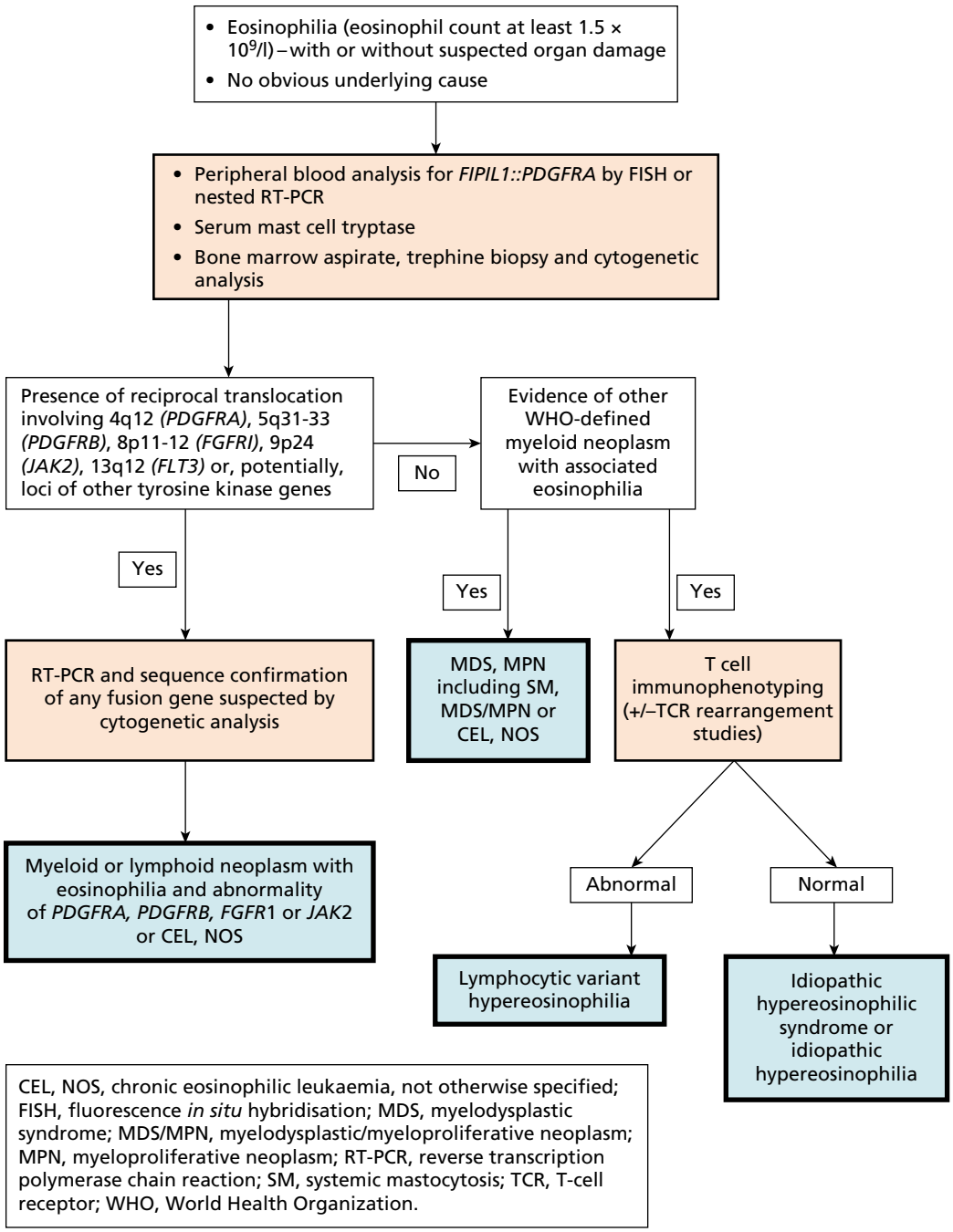


Figure 35.16 Suggested algorithm for suspected clonal eosinophilia (Source: Butt *et al.* 2017/John Wiley & Sons).

this section is devoted to discussing the management of eosinophilia related to haematological causes and hypereosinophilic syndrome. For all of these conditions stem cell transplantation should be considered for those patients with refractory disease or progressive organ damage.

Treatment of clonal eosinophilia

For those patients with an identified targetable abnormality an appropriate drug should be selected. The best-described abnormality is clonal eosinophilia with *FIP1L1::PDGFRA* (which can rarely also present with acute leukaemia). These

patients are frequently male and should be treated with low-dose imatinib. Specialist advice should be sought and monitoring of clonal burden will facilitate personalised therapy. For some patients, there is an emerging literature suggesting it is possible to stop the imatinib after some years of treatment. This is a field where variants and targets are expanding. Other examples include *PDGFRB* rearrangement or *ETV6::ABL1* fusion whom should receive imatinib. *ETV6::FLT3* fusion should be considered for sunitinib or sorafenib therapy. Those with *JAK2* rearrangement should be considered for ruxolitinib therapy. Recently for the rare patients with 8p11 or *FGFR* rearrangements pemigatinib has shown dramatic responses. It is recommended that patients with other haematological neoplasms including AML with clonal eosinophilia should have treatment directed at management of the neoplasm. If there is organ damage or dysfunction relating to the eosinophilia, treatment with corticosteroids should also be given.

Chronic eosinophilic leukaemia (CEL NOS)

CEL, NOS is *BCR::ABL1*-negative without detectable clonal markers known to be associated with eosinophilia and an eosinophil count of at least $1{\cdot}5 \times 10^9$/L. As distinct from AML-Eo, blast cells must be increased but less than 20% in both the peripheral blood and bone marrow. Overall CEL, NOS is recognised to be a rare and aggressive disorder associated with a median survival of 20 months and a high rate of acute transformation.

Treatment of lymphocytic variant of hypereosinophilic syndrome

Patients with the lymphocytic variant of hypereosinophilic syndrome (HES) can be managed in the same manner as idiopathic HES once an underlying lymphoma has been excluded.

Treatment of idiopathic hypereosinophilia and idiopathic hypereosinophilic syndrome (HES)

Idiopathic hypereosinophilic syndrome is defined by the presence of an eosinophilia, absence of an identified cause and detectable tissue damage typically including the heart, lungs, skin, nervous system, GI tract and thrombosis. In the absence of detectable tissue damage the diagnosis is idiopathic hypereosinophilia which for the most part does not require treatment.

Patients with idiopathic HES requiring treatment should be treated in the first instance with corticosteroids, high doses may be required. For those HES patients who either do not respond adequately to corticosteroids, or who either require prolonged corticosteroid therapy or are intolerant of corticosteroids other agents should be considered but these are patients who should be discussed with a specialist centre. A short trial (4–6 weeks) of any of the following could be considered: imatinib, immunomodulatory agents (interferon alpha, ciclosporin or azathioprine), myelosuppressive therapy (hydroxycarbamide) and finally alemtuzumab. Note should be made of the efficacy of monoclonal antibody therapy for example with mepolizumab (anti-interleukin 5).

Chronic neutrophilic leukaemia

Chronic neutrophilia is most commonly secondary, for example to chronic infections, inflammation or malignancy, with mildly elevated counts also found in smokers or corticosteroid use. Clonal causes include chronic myeloid leukaemia and the WHO 2022-defined condition MDS/MPN with neutrophilia (formerly 'atypical CML'), both of which are associated with left shift in the peripheral blood, and CNL, characterised by expansion of predominantly mature neutrophils.

Clinical features

CNL is a rare neoplasm whose true incidence is unknown, particularly when strict diagnostic criteria are applied. Median age at diagnosis is approximately 66 years. Patients may present with constitutional symptoms, splenomegaly and, in some cases, anaemia or thrombocytopenia in addition to the neutrophilia. The blood film shows an increase in segmented neutrophils and bands (Figure 35.17), the bone marrow is hypercellular with marked granulocytic expansion and without dysplasia or increased blasts, and there is no peripheral monocytosis. Diagnostic criteria (Table 35.17) now include the presence of a *CSF3R* mutation and if absent, the diagnosis should be made with care and secondary causes fully excluded.

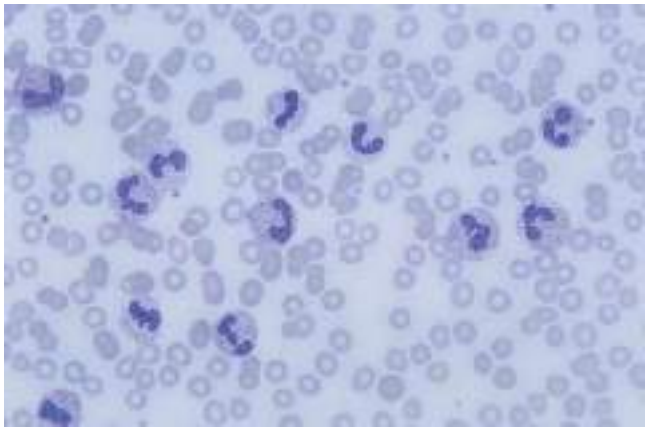


Figure 35.17 Abundance of mature neutrophils and band forms in a blood film from a patient with chronic neutrophilic leukaemia.

Table 35.17 Diagnostic criteria for chronic neutrophilic leukaemia from the World Health Organization (2022).

1	Peripheral blood white cell count $\geq 25 \times 10^9$/L
	Segmented neutrophils + band forms ≥80% of white blood cells
	Neutrophil precursors (promyelocytes, myelocytes, metamyelocytes) <10% of white blood cells
	Myeloblasts rarely seen
	Monocytes count <10% of peripheral blood leucocytes and not meeting criteria for CMML
	No dysgranulopoiesis
2	Hypercellular bone marrow
	Neutrophil granulocytes increased in percentage and number
	Neutrophil maturation appears normal
	Myeloblasts <5% of nucleated cells
3	Not meeting criteria for *BCR::ABL1*+ CML, PV, ET or PMF
4	No rearrangement of *PDGFRA*, *PDGFRB* or *FGFR1* or *PCM1::JAK2*
5	Presence of *CSF3R* T618I or other activating *CSF3R* mutation
or	
In the absence of a *CSF3R* mutation, persistent neutrophilia (≥3 months), splenomegaly and no identifiable cause of reactive neutrophilia (including absence of plasma cell neoplasm) or, if present, demonstration of clonality of myeloid cells by cytogenetic/molecular studies.	

Treatment

No therapies have been shown to improve survival, with the exception of allogeneic stem cell transplantation which is appropriate for the small minority of younger patients. Hydroxycarbamide has been used most frequently; other options include interferon, hypomethylating agents and busulphan. Responses to ruxolitinib have been reported in some patients with the *CSF3R T618I* mutation, although patients with a compound mutation (co-existing truncation mutation) are likely to be resistant.

Prognosis

Median survival of CNL is approximately 2 years, with a proportion of patients transforming to AML. *ASXL1* mutations confer an adverse prognosis. However, it is important to remember that this entity like many of those described in this chapter is very uncommon and further data is required to understand likely outcomes for patients and optimise treatment approaches.

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CHAPTER 36

Macrophages, dendritic cells and histiocytic disorders

36

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Origin and classification

Overview

Macrophages and DC are myeloid cells that play fundamental roles in tissue homeostasis, immunoregulation and host defence. They are found in nearly all healthy tissues where they maintain a state of immune tolerance. Resident macrophages are also known as histiocytes – literally 'tissue cells' – a term that usefully distinguishes them from monocyte-derived cells that enter from the blood. During inflammation, monocyte-derived cells are recruited to tissues, massively augmenting the population of macrophages. DC migrate to draining lymph nodes to prime naïve T cells and direct immune responses. Both lineages contribute to the afferent phase of an immune response by elaborating chemokines, cytokines and other mediators to attract leukocytes from the bloodstream. While DC play a primary role as professional antigen-presenting cells in the lymph nodes, inflammatory monocyte-derived cells are also able to present antigen to primed T cells, modulating and amplifying local immune responses. Recruited inflammatory monocytes differentiate into a range of activated macrophage phenotypes with important effector roles in the phagocytosis and killing of pathogens, recognition of tumours, efferocytosis of apoptotic and dead cells and resolution of inflammation.

A modern view of macrophage and dendritic cell ontogeny

Recent developments have taken our understanding of macrophages and DC far beyond the mononuclear phagocyte system in which macrophages and DC are derived from monocytes emanating from the bone marrow.

Macrophages

Mammalian haematopoiesis develops through successive waves beginning extra-embryonically in the yolk sac blood islands whence primitive macrophages migrate through the pre-vascular embryo and establish themselves in most tissues including the brain and epidermis (Figure 36.1a). A wave of fetal liver monocytes then enters the tissues significantly augmenting the population of resident macrophages, with the exception of microglia, now isolated by the blood–brain barrier. At least in animal models, microglia remain exclusively yolk sac-derived for life and together with most other populations including Langerhans cells, Kupffer cells and alveolar macrophages, form stable and self-renewing populations into adulthood. Other macrophage populations in the gut, skin and interstitial tissues continue to recruit from the monocyte pool derived from definitive haematopoietic stem cells (HSC) via chemokine receptor CCR2 which binds the monocyte chemoattractant proteins, CCL2, CCL8 and CCL16.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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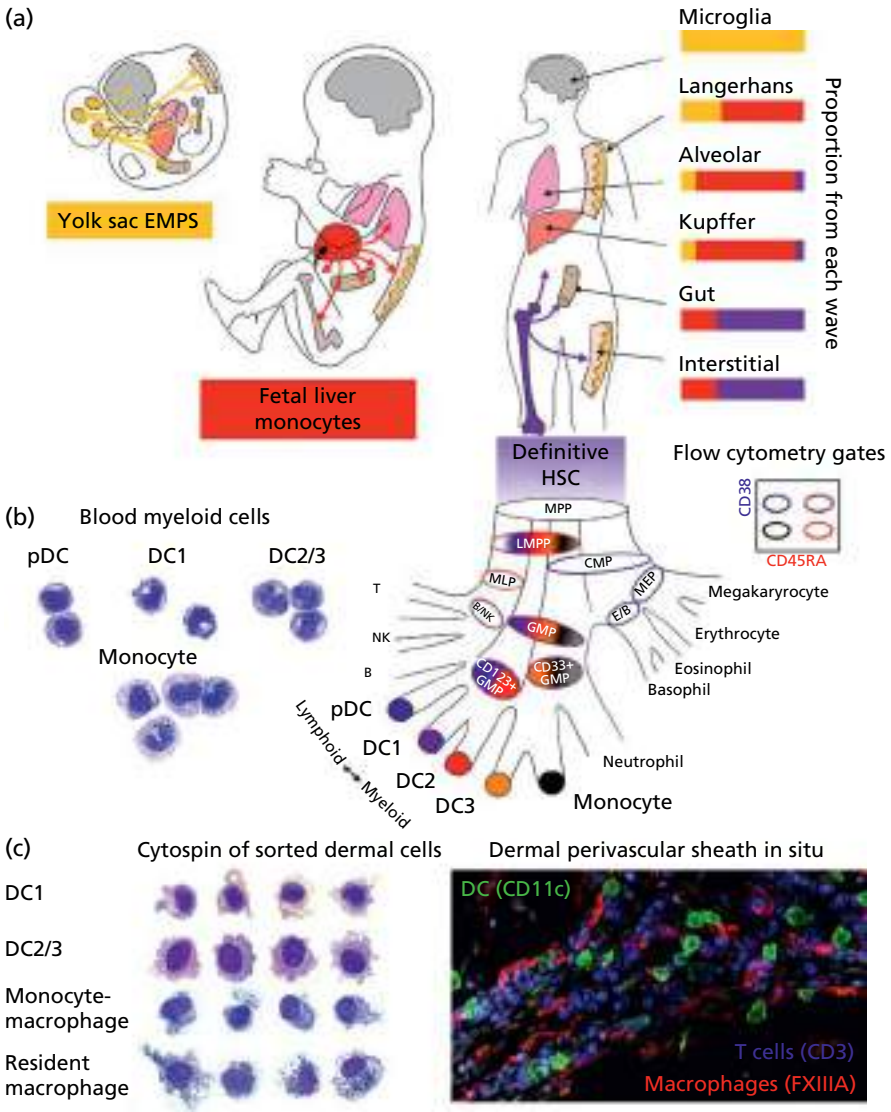

Figure 36.1 (a) Schematic depicting three waves of macrophage development described in the mouse from yolk sac early myeloid progenitors (EMP; yellow), fetal liver monocytes (red) and definitive haematopoieic stem cells (HSC; purple). Bars on the right-hand side show the estimated contribution of each wave based on mouse data redrawn from Bleriot, 2020. (b) Lefthand side: Geimsa-stained cytopsins of purified human blood DC and classical monocytes (see Collin, 2018 for details). Righthand side: schematic showing the development of human DC from lymphoid-primed multipotent progenitors (LMPP), via granulocyte-macrophage progenitors (GMP) according to contemporary lineage-primed models of haematopoiesis. MLP: multilymphoid progenitors: B/NK progenitors of B and NK cells; CMP common myeloid progenitors; MEP megakaryocyte erythryroid progenitors; E/B: eosinophil and basophil progenitors. The inset is a key to the surface phenotype of each progenitor compartment. (c) Lefthand side: Geimsa-stained cytopsins of purified human tissue DC and macrophages from single cell suspensions of dermis. Righthand side whole-mount immunofluorescence of human dermis showing DC, macrophages and T cells in a perivascular sheath. (Source: Image courtesy of Xiao Wang).

Dendritic cells

Dendritic cells are now known to have an independent haematopoietic origin to monocytes and macrophages. The lineage boundary only becomes blurred when inflammatory monocytes become potent antigen-presenting cells corresponding to the classic monocyte-derived DC-derived in vitro with GM-CSF and IL-4. DC traffic continuously through tissues to the draining lymph nodes where they survive for a few weeks at most. At the onset of inflammation, DC undergo functional maturation with increased expression of MHC class II, co-stimulatory molecules (CD80, CD83 and CD86) and the chemokine receptor CCR7 that directs their homing to lymph nodes along CCL19 and CCL21

chemokine gradients. Migratory DCs from inflamed tissues transport antigen to T cell areas of the lymph node to initiate immune responses.

Monocyte and DC haematopoiesis

In both human and mouse systems, it is possible to identify discrete pathways of haematopoiesis for monocytes and DC. Haematopoiesis is now conceived as a collection of discrete trajectories primed early in development, rather than a sequence of bifurcating fate decisions (Figure 36.1b). Precursors such as the granulocyte-monocyte precursor (GMP) are not discrete homogenous populations but regions of phenotype parameter space containing related lineage trajectories. As lineages develop they gradually become more distinct, until it is possible to separate them into mature cell phenotypes. The textbook dichotomy, splitting haematopoiesis into lymphoid and myeloid lineages, has been revised; it is now thought that all mononuclear cells emerge from a common lympho-myeloid trunk (lymphoid-primed multipotent progenitors; LMPP). Thus, it is no longer surprising that DC appear to differentiate from progenitor fractions bearing both lymphoid and myeloid markers.

Classification of macrophages and dendritic cells

Immune cell lineages are highly diversified to provide a wide range of finely tuned responses. Macrophages are geographically specialised by transcriptional programmes specific to different tissue niches. Within each tissue, the specialised populations of microglia, Langerhans cells, alveolar macrophages and Kupffer cells are complemented by neuronal and vascular-associated resident macrophages and transient populations of monocyte-derived macrophages. The classification of mammalian DC is now well established. Two major classes exist, myeloid DC, bearing CD33, CD13, CD11b and CD11c in common with monocytes, and plasmacytoid DC which lack these antigens but express the naïve T cell marker CD45RA and high levels of the IL-3 receptor CD123. Myeloid DC comprise three subsets DC1, DC2 and DC3. DC1 (previously known as cDC1 for conventional DC1) expresses the lectin CLEC9A for apoptotic cell uptake and the unique chemokine receptor XCR1. DC1 is characterised as cross-presenting DC critical for responses to virus-infected cells, intracellular pathogens and tumours. DC2 and DC3 are closely related and were previously known collectively as cDC2. These DC correspond to the 'indeterminate cell' that inhabits interstitial tissues, expressing lower CD1a than LC and no Langerin. They both express CD1c, CD2 and FcεR1 and are more abundant than DC1 in blood and tissues. DC2 express some antigens found on DC1, such as BTLA, while DC3 share monocyte antigens including CD14 and CD163. DC3 have greater expression of inflammatory gene modules and are selectively expanded in inflammatory and autoimmune diseases. Both are capable of presenting antigens to T cells. By transcriptional analysis DC can be aligned along a lymphoid-myeloid axis in the order: pDC; DC1; DC2; DC3 (Figure 36.1).

Monocyte and dendritic cell deficiency

It is well known that inborn errors of haematopoiesis can lead to cellular deficiency such as anaemia, neutropenia or thrombocytopenia. Recently, a number of constitutive genetic defects have been described that lead to the loss of monocytes and DC and comprise a class of primary immunonodeficiency. Heterozygous *GATA2* mutation truncates the entire lymphomyeloid branch of haematopoiesis causing a severe loss of DC, monocytes, B and NK cells associated with susceptibility to mycobacterial infection, papilloma virus-driven malignancy and pulmonary alveolar proteinosis. Acquired *ASXL1* mutation and monosomy 7 are common leading to myelodysplasia and acute myeloid leukaemia. *IRF8* is a critical gene regulating monocyte and DC production in an exquisitely dose-dependent manner. In biallelic deficiency of *IRF8*, there is severe monocytopenia, loss of DC and an excess of neutrophils. Less severe defects ablate only DC development causing an excess of monocytes or DC3. Ikaros (*IKZF1*) mutation also affects the balance of haematopoietic output between pDC and DC1. Recently human Flt3 Ligand *(FLT3LG)* mutation has also been described as a cause of DC deficiency. The presence of Langerhans cells and some tissue monocytes in these disorders demonstrates their independence from the bone marrow in humans.

The histiocytic neoplasms

Overview

Classification

The histiocytic neoplasms are a group of rare and complex disorders classified by the Histiocyte Society into five clinicopathological groups: L, C, R, M and H (Table 36.1). These groups do not map to WHO classification or ICD codes and in the near future, more rigorous genetic profiling will doubtless instigate another revision. This chapter will describe the major clinically recognisable disorders known to arise from acquired genetic aberrations of mitogen-activated protein kinase (MAPK) and PI3K–AKT pathways: Langerhans cell histiocytosis (LCH); Erdheim Chester Disease (ECD) Juvenile Xanthogranuloma (JXG) and Rosai Dorfman Destombes Disease (RDD). Haemophagocytic lymphohistiocytosis (HLH) is a completely distinct clinical and pathological process that will be described separately.

Molecular and cellular aetiology

In 2010, the oncogenic driver $BRAF^{V600E}$ was identified in about two-thirds of cases of LCH, revolutionising the field of

Table 36.1 Classification of the histiocytic disorders.

Histiocyte society		Common genetics	Markers	WHO	ICD11 2B31
L	**LCH**	*BRAF*^{V600E} *BRAF* indel *BRAF* fusion *MAP2K1*	S100 CD1a Langerin	LCH	2B31.2
	ECD	*NRAS, KRAS* *PIK3CA* *ARAF* *BRAF*^{V600E} *MAP2K1*	CD14 CD68 CD163 FXIIIA +/−	ECD	2B31.Y
	ICH ALK	*ETV3-NCOA2* *KIF5B-ALK* *Other-ALK*	CD1a CD14 CD68 CD163		2B31.6
C	**JXG**	*CSF1R* *NRAS, KRAS* *BRAF* fusion *MAP2K1* *NTRAK* fusion *RET* fusion	CD14 CD68 CD163 FXIIIA fascin	JXG	2B31.0
	Adult XG*	Unknown	CD68 CD163		
R	**RDD**	*NRAS, KRAS* *MAP2K1* wild-type	S100 CD68 CD14+/− CD163+/−		2B31.Y
M	HS LCS IDCS FDCS FRCT	Complex		HS LCS IDCS FDCS FRCT	2B31.1 2B31.2 2B31.4 2B31.5 2B31.7
H	HLH	Germline	CD14 CD68 CD163		

histiocytosis. This remains the most prevalent mutation overall but has been joined by a wide catalogue of hits to the MAPK pathway including *BRAF* indels and fusions, *MAP2K1* (MEK) mutations, common oncogenic mutations of *NRAS* and *KRAS* and most recently, mutation of the M-CSF receptor gene *CSF1R*. Some cases, especially of RDD, may yet turn out to be reactive phenomena, but it remains likely that all classic manifestations of these conditions are driven by somatic mutation. New potential driver mutations are still being documented. *ALK* translocations, most frequently *KIF5B-ALK,* define a new pathological entity of ALK+ histiocytosis. *ETV3-NCOA2* translocation has been documented as the driver of indeterminate cell histiocytosis.

MAPK pathway activation

The MAPK mutations driving histiocytic neoplasms beautifully underscore the role of M-CSF in promoting macrophage and DC development and neatly parallel mutation of the JAK/STAT signalling pathway driving conventional myeloproliferative neoplasms (MPN) (Figure 36.2a). Unlike conventional MPN, however, MAPK pathway activation, confers little proliferative advantage on HSC and is only found at very low frequency in HSCs even in multisystem disease. In single-system disease, mutation is localised exclusively to the peripheral tissues. Lesions progress through the accumulation of neoplastic histiocytes with relatively low proliferative potential. Histiocytic neoplasms do not typically progress to myeloid

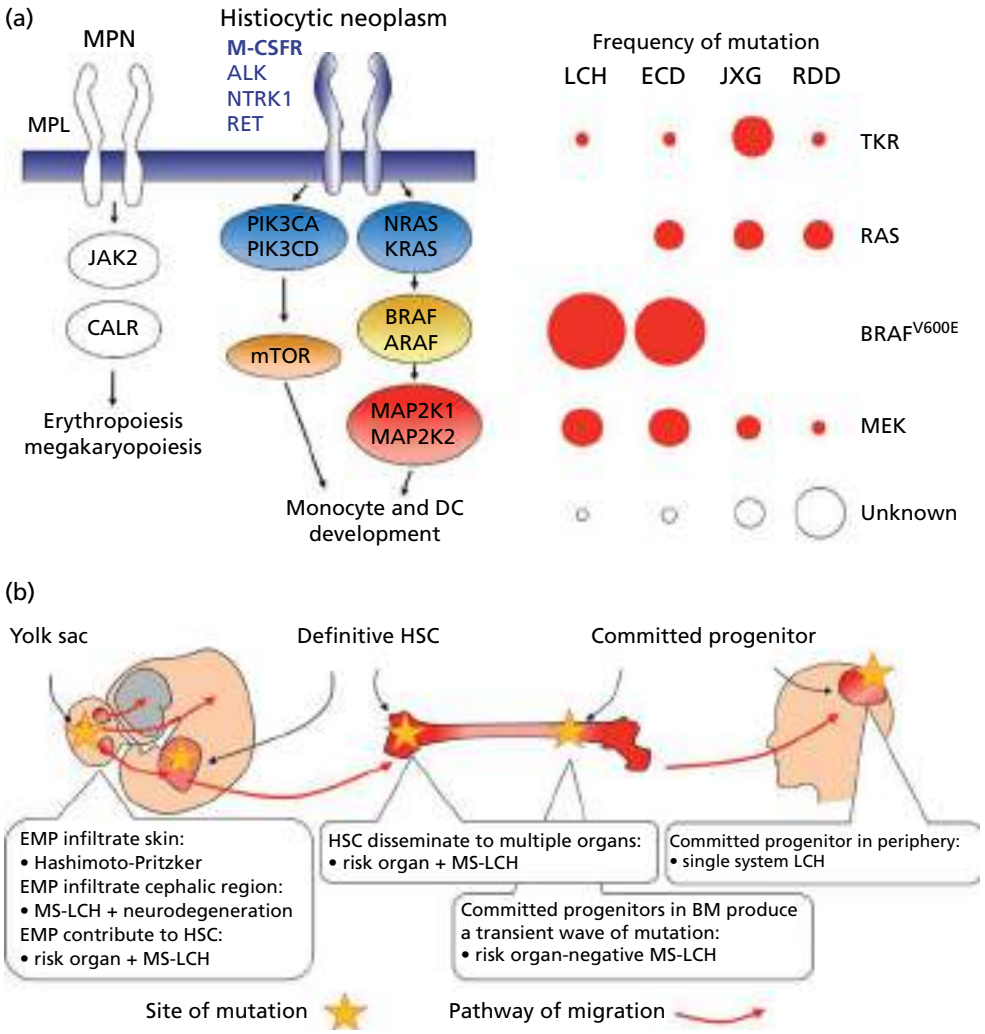

Figure 36.2 (a) Schematic comparing signal transduction activation in myeloproliferative neoplasms (MPN) and MAPK pathway activation in histiocytic neoplasms. Most commonly mutated proteins are labelled in bold. Red circles depict the relative frequency of mutation at each level in the pathway in each histiocytic neoplasm. (Source: Data redrawn from Durham, 2019). (b) Multistage model of LCH pathogenesis depicting how mutation arising at different stages of haematopoiesis might influence the clinical spectrum of disease. The involvement of early myeloid progenitors (EMP) in LCH is entirely speculative based on mouse models. HSC are known to be involved in risk organ-positive multi-system (MS)-LCH but not in most instances of risk-organ-negative or single system-LCH.

malignancy but conversely, may evolve as a subclone of clonal haematopoiesis and myelodysplasia.

Second hits

Second hits may explain the peculiar tissue tropism of many histiocytic neoplasms. LCH localises to the skin folds in infants, dental alveolar bone in adults and lungs in smokers, which are all sites of inflammation. ECD and xanthomatous histiocytic disorders have predilection for skin, internal adipose tissue and vasculature where lipid metabolism may produce pro-inflammatory mediators. Critically, the MAPK pathway is a rheostat for response to inflammation at the convergence of somatic mutation and exogenous stimuli. In keeping with this, case-control studies have reported that neonatal infection was associated with an increased risk of multi-system LCH (OR 2.8–3.8).

Genetic analysis

Genetic analysis has become an important new tool in the diagnosis, prognosis and treatment of these complex pathologies. In localised disease, mutation is often confined to the lesion but in multi-system LCH and advanced ECD, mutation is usually accessible from myeloid cells and cell-free DNA in the blood. The detection of mutation in the blood is therefore of potential value in staging and prognosis. The identity of the mutated gene

is also relevant. In paediatric LCH, *BRAF*^{V600E} is present in 90% of infants with multi-system LCH and is associated with an increased risk of relapse and neurodegeneration. *BRAF* mutation is also more prevalent in certain subgroups of ECD. The discovery of a molecular aetiology has had profound implications for therapy since many drugs are available targeted to *BRAF* and *MEK*, owing to the prevalence of *BRAF* mutation in melanoma and other cancers.

Langerhans cell histiocytosis

LCH epidemiology

The population incidence of LCH in the UK is 1.5 per million person-years ranging from 11.9 in infants <1 year old to 1.0 in those >15 years. An equal number of children and adults are diagnosed each year. North America, France and Sweden, report similar incidences in children ranging between 2.6 and 8.9 new cases per million. Social deprivation is associated with an increased risk of LCH independent of smoking which is the primary trigger for pulmonary LCH.

LCH pathophysiology

LCH is characterised by intense inflammatory lesions containing neoplastic histiocytes expressing S100, CD1a and langerin (CD207, a C-type lectin) admixed with a background of eosinophils, macrophages, multinucleated giant cells and T cells (Figure 36.3). Bone lesions with this morphology are synonymous with 'eosinophilic granuloma'. In other sites, notably the bone marrow liver and brain, LCH cells are rare. LCH-associated neurodegeneration is typically a gliotic process involving T cells. By electron microscopy, LCH cells contain Birbeck granules, unique endosomal structures found

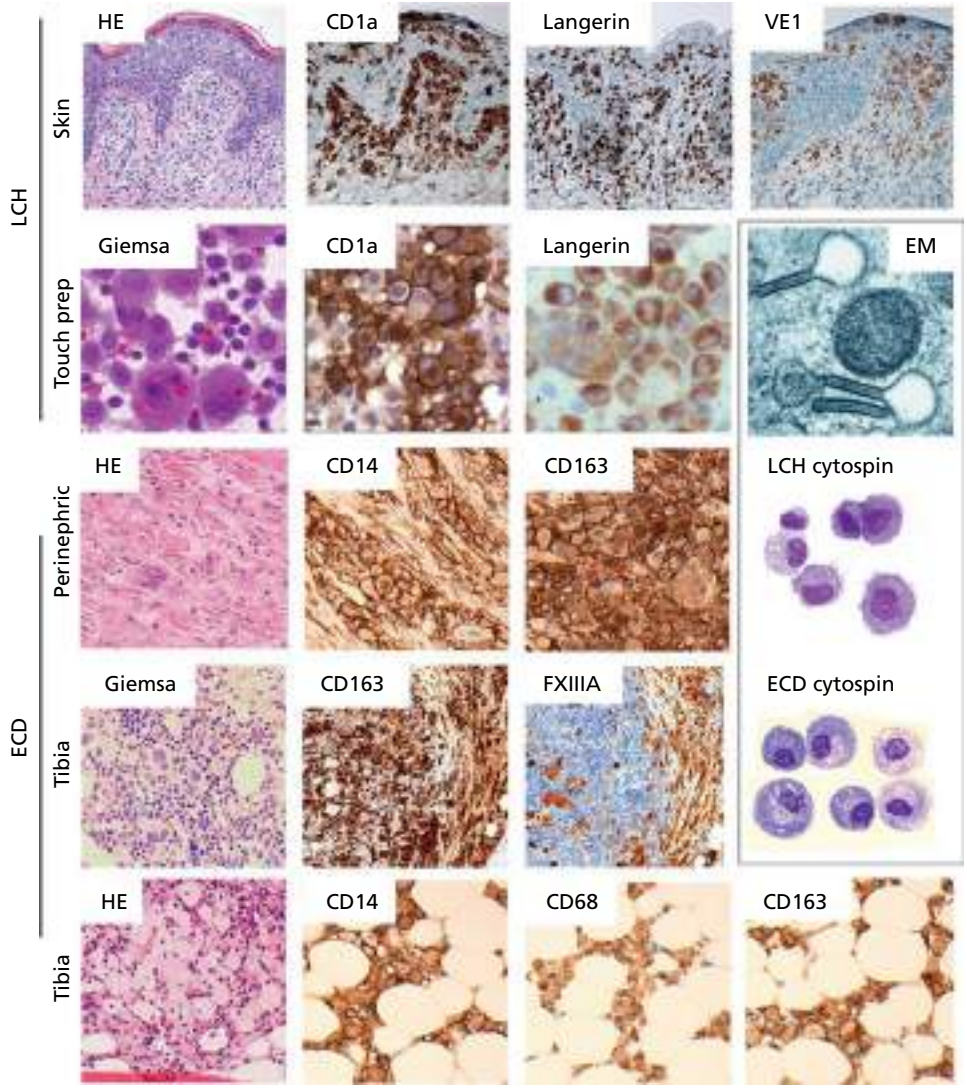

Figure 36.3 Histology, touch prep images and electron microscopy. (Source: Courtesy of Marian Malone).

in Langerhans cells. By gene expression, LCH cells are relatively immature and express a high level of PDL-1, holding T cells in an anergic state. This is reflected in their relatively bland pale-staining cytoplasm and the famous 'coffee bean' nucleus containing folded but open chromatin. LCH can cause a wide range of pathology from single lesions to disseminated high-risk disease (Figure 36.2b). Single system disease appears to arise either locally through mutation of Langerhans cells or tissue macrophages, with self-renewal capacity, or from the bone marrow through mutation of committed progenitors producing a transient wave of abnormal haematopoiesis. In multi-system disease, somatic mutation is detectable in HSC, consistent with disseminated disease and the propensity for relapse after treatment. DC2, DC3 and monocytes all harbour *BRAF* mutation and are able to differentiate into high langerin-expressing cells with Birbeck granules, *in-vitro*. A third possibility is that mutations could arise prenatally in early yolk sac or fetal liver myeloid progenitors and seed the microglia of the brain, cutaneous Langerhans cells and HSC. This mechanism has been proposed for congenital self-healing LCH (Hashimoto–Pritzker disease), infantile multisystem LCH and LCH-related neurodegeneration. Congenital mosaicism amongst histiocytes might explain regional cephalic disease involving the scalp, cranio-facial bones, posterior pituitary and neurodegeneration.

LCH clinical presentation

The clinical presentation of LCH is extremely variable potentially involving many different organs (Figure 36.4). A simple staging system distinguishes between single-system disease which is usually confined to either skin, bone or lungs (in adults) and multisystem disease which can be severe when it involves the bone marrow, liver and spleen. These sites are therefore known as 'risk organs' leading to the designation risk organ positive (RO+) LCH. RO+ multi-system LCH occurs almost exclusively in infants and young children and matches the historical syndrome of Letterer Siwe disease. The major acute consequences are fever, cytopenia, intrahepatic jaundice and liver failure. These elements, together with organomegaly, area of skin involvement, lymphadenopathy, bone and soft tissue lesions have been compiled into a disease activity score (DAS). RO+ multi-system LCH may be amplified by secondary HLH, considerably increasing the therapeutic challenge. Prior to molecularly targeted therapy, RO+ multi-system LCH incurred a mortality of 10% and a high risk of late effects including neurodegeneration. This type of presentation is not described in adults and cytopenia or severe systemic illness should prompt a thorough investigation for Erdheim Chester disease concomitant myelodysplasia or other myeloid malignancy.

Risk organ negative (RO−) multisystem LCH has many potential variants of two or more organs. The classic triad of exophthalmos, ulcerating skull lesions and diabetes insipidus has been recognised for many decades as Hand Schuller Christian disease, also described as 'chronic multifocal LCH'. The lytic skull lesions are contiguous with soft tissue inflammation and painful skin ulceration. Lesions may extend to the mastoid and auditory canal, maxilla and mandible presenting as ear discharge and floating teeth. LCH is thus an important rare differential of otitis externa and periodontitis. Involvement of facial bones, base of skull and posterior pituitary have well-recognised associations with neurodegeneration. Lesions at the base of skull are known as 'special sites' for the risk of neurodegeneration, leading to more intensive treatment regimens in children.

Single-system bone LCH has a predilection for the axial skeleton and may be unifocal or multifocal. Eosinophilic granuloma of a vertebral body can present with a sudden onset of back pain and vertebra plana. Skin rashes frequent the skin folds and are typically erosive, itchy and very painful but provide an easy biopsy site. Their appearance can be misleading and overlap with nappy rash in infants or eczema and sebbhorreic dermatitis in older children and adults.

Pulmonary LCH occurs in two completely different clinical settings: neonates, with severe single organ respiratory failure or as part of RO+ multisystem LCH; and adolescents and adults who smoke, where it is mostly single system but can have multisystem extrapulmonary manifestations. Presenting features are breathlessness, chest pain and pneumothorax. Radiology reveals cysts at the apices, which may be very large, with widespread nodules and interstitial inflammation typically sparing the lung bases. A radiological diagnosis is usually reached without biopsy.

LCH can infiltrate the posterior pituitary, present as mass lesions and cause a devastating neurodegenerative syndrome. Pituitary stalk inflammation, swelling or small masses are frequently seen with recent diabetes insipidus. Mass lesions can cause headache, visual disturbance and gross derangement of satiety, memory and executive function. Hyperphagia is an uncommon presentation of hypothalamic lesions that, with memory loss, can prove unmanageable and ultimately fatal. Neurodegeneration is suspected when educational performance declines and difficult behaviours appear, especially with the onset of gait disturbance, clumsiness, dysarthria and dysphagia indicative of cerebellar dysfunction. More than half of patients with diabetes insipidus or craniofacial bone disease develop high T2-weighted signal in the cerebellum within 3 years, and one-quarter of these (about 15% overall) suffer irreversible neurological decline.

LCH diagnosis

Several recent guidelines describe the diagnosis and management of LCH in detail (see further reading). A thorough clinical history and examination are required to document the extent of disease. Bone pain, dental health, ear discharge, polyuria and polydipsia, skin rash, breathlessness and neurological symptoms are all important features. The extent of skin involvement, bone tenderness, chest signs, organomegaly, lymphadenopathy, jaundice, ascites and bruising should be noted. A full blood workup is usually indicated to exclude cytopenia, liver dysfunction, endocrine

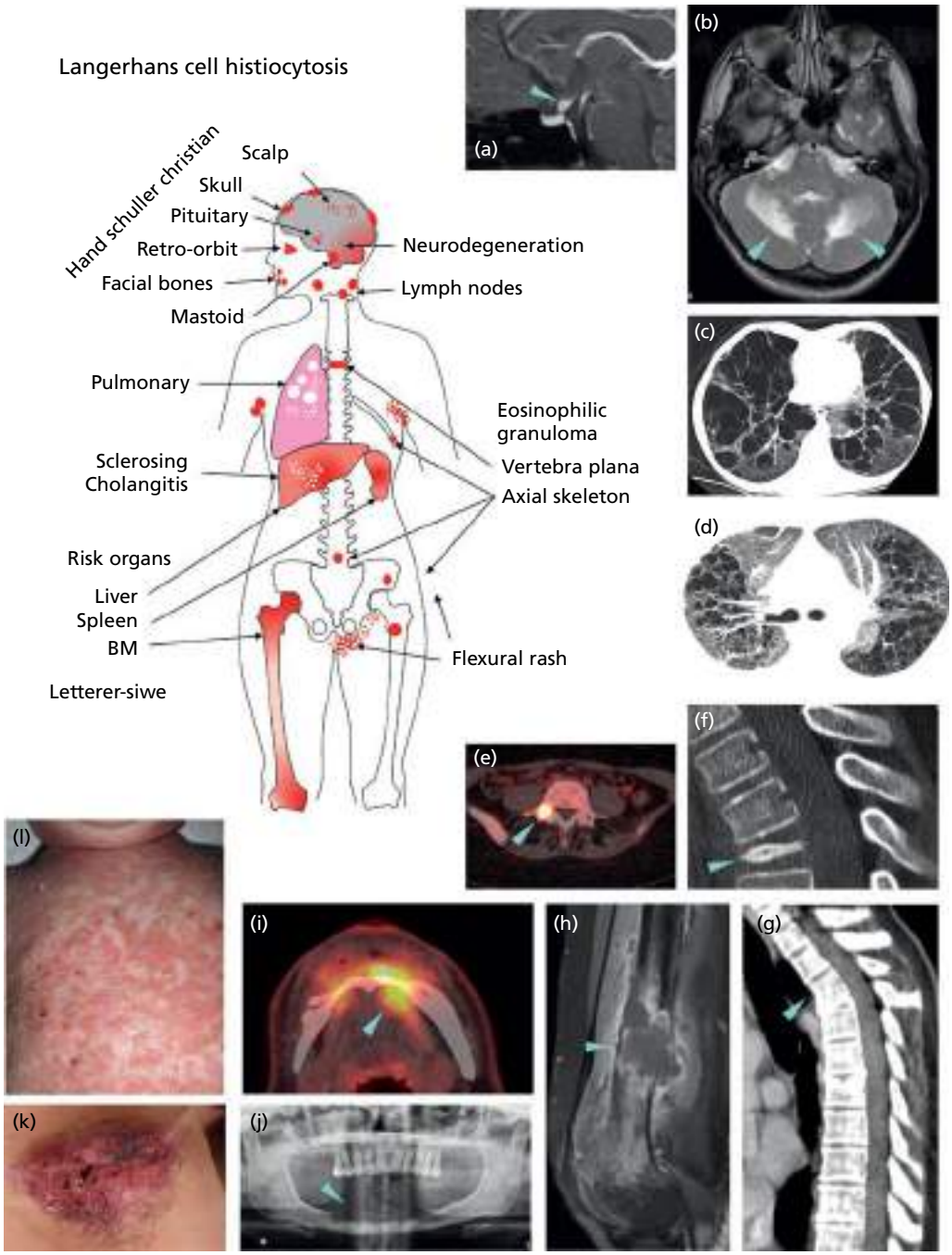

Figure 36.4 Clockwise from top right: (a) Contrast MRI pituitary stalk enhancement. (b) MRI T2 high signal in the dentate nuclei. (c) Pulmonary LCH in an infant. (d) Pulmonary LCH in an adult. (e) Eosinophilic granuloma L5. (f, g). Vertebra plana. (h) Massive eosinophilic granuloma presenting as fractured femur. (i, j) Destruction of the mandible on PET-CT and orthopantogram. (k) LCH rash in an adult axilla. (l) LCH rash in an infant. (Source: Courtesy of Dr. Carl Allen).

abnormalities and hyperinflammation/HLH. Plain radiography is helpful for sites of bone pain. Most patients warrant at least one PET-CT to fully document sites of disease although skull lesions can be difficult to visualise adjacent to the brain, and PET avidity may be relatively low for older bone lesions and liver involvement. MRI of the head is useful to document pituitary stalk and posterior bright spot together as a baseline for neurodegeneration. Clinical evaluation includes lung function, water deprivation for suspected diabetes insipidus, ataxia rating, mental state including memory and depression scores. Pathological diagnosis

is usually reached by skin or bone biopsy; occasionally, lymph node, gut, liver or lung biopsy is necessary. Bone marrow examination is indicated with pancytopenia and is essential for defining RO+ status. A full molecular evaluation should be undertaken involving a rapid and sensitive PCR-based test for *BRAF*^{V600E}. In wild-type cases, DNA sequencing should be undertaken for other MAPK pathway mutations or deletions and RNA should be analysed for gene fusion. These investigations are increasingly useful for confirmation of difficult pathology and inform the decision for targeted therapy in refractory disease.

LCH management

International treatment protocols, devised over the last 40 years, deliver risk-adjusted therapy to patients with LCH. Unifocal bone LCH often resolves after biopsy or with intra-lesional corticosteroid. Wide excision or resection should be avoided, as severe defects often regenerate and unnecessary grafts and prostheses often lead to severe chronic pain. Post-inflammatory pain is common and may respond to indometacin or bisophosphonates. In adults, fractionated radiotherapy in the range 10-20Gy is highly effective and can be useful for critical, painful or inaccessible bone lesions, although it is seldom used for children owing to growth arrest of the involved field. Skin disease often responds to topical immunomodulation by corticosteroids or adjuvants.

The mainstay of frontline systemic therapy for multi-system LCH in children is vinblastine and prednisolone, although RO+ disease has a high relapse rate. Salvage with cytarabine, cladribine or clofarabine is required for nearly half of RO+ infants, initially exposed to vinblastine. Cytarabine and cladribine are also useful for older adults with multifocal bone disease, pulmonary LCH or multisystem LCH who do not tolerate vinblastine and prednisolone well. Etoposide is active in LCH but rarely used owing to the risk of t-AML.

Maintenance plays an important role in treating LCH. Daily 6-mercaptopurine and weekly methotrexate are used for 12–24 months in paediatric protocols and for adults. Azathioprine or 6-mercaptopurine has also been widely used for skin disease in adults. Hydroxycarbamide is known to suppress chronic relapsing skeletal and cutaneous disease.

MAPK inhibitors have revolutionised salvage for children and adults with the highest risk of disease who fail conventional chemotherapy. Most experience has been obtained with vemurafenib (targeted to *BRAF*^{V600E}), dabrafenib (*BRAF*), cobimetinib and trametinib (MEK inhibitors). For patients with BRAF mutation, a combination of *BRAF* and MEK inhibitors may be used. If *BRAF* is wild-type or the genotype is unknown then MEK inhibition is usually effective. An important limitation is that it is difficult to withdraw therapy without brisk relapses. Sustained MAPK inhibitor therapy is not without difficulty owing to the many idiosyncratic toxicities of these drugs and unknown long-term consequences.

Pulmonary LCH requires absolute abstinence from smoking or may progress to end-stage respiratory failure requiring lung transplantation. Systemic treatment with vinblastine and prednisolone, cytarabine or cladribine is effective in progressive disease or with concurrent extra-pulmonary involvement, although cytoreductive treatment is very challenging to deliver in the face of declining lung function. MAPK inhibitors are yet to be widely used but may play a key role in patients with severe compromise unable to tolerate chemotherapy.

Neurodegeneration was considered an irreversible and ultimately fatal complication of LCH, before the advent of MAPK inhibitors. There was evidence that cytarabine or intravenous immunoglobulin could modify progression but neither was associated with dramatic improvement as has been documented with inhibitor therapy.

Fortunately, in children, overall survival is good and only patients with RO+ disease are at risk of death (up to 5%). However, late effects and permanent disability are common in patients treated for multi-system disease. Children face multiple challenges of normal development and educational achievement that have long-lasting consequences. In adulthood, chronic skeletal pain, respiratory compromise, anxiety and depression are commonplace and difficult to manage. The burden of morbidity in older adults is starkly illustrated by their poor survival of less than 40% at 5 years.

Erdheim Chester disease

Erdheim Chester disease epidemiology

ECD is the most clinically important 'non-Langerhans' histiocytic neoplasm. It is about half as frequent as LCH with an estimated annual population incidence of 0.5 per million, a mean age at diagnosis of 55 years and male preponderance of 3:1.

ECD pathophysiology

ECD is characterised by inflammatory, fibrotic lesions containing xanthomatous (fat-laden) foamy macrophages expressing typical macrophage markers: CD68, CD14, CD163 and variable factor XIIIA (Figure 36.3). Touton giant cells are often but not invariably found. ECD, JXG and other xanthomas are referred to as 'non-Langerhans' histiocytosis because the abnormal histiocytes are negative for LCH markers, S100, CD1a and langerin. Critically for the pathologist, ECD macrophages have no specific neoplastic phenotype and it is not possible to separate ECD from many other xanthomatous and non-neoplastic disorders. The cellular background is also highly variable and may be mimicked by a wide range of pathology. The common molecular aetiology of histiocytic neoplasia is underscored by the overlap between pathological LCH and ECD in about 10% of patients and the observation that LCH lesions evolve with time to become more fibrotic and 'ECD-like'. A distinct entity overlapping with Rosai Dorfman disease has also been described.

In simple terms, ECD and LCH are respectively macrophage-biassed and DC-biassed evolutions of a shared neoplastic process. Mutations are concentrated in monocytes and myeloid DC

similarly to multi-system LCH but the relationships between abundance of alleles, stage of disease and response to therapy are less well established. About half of ECD is caused by *BRAF*^{V600E}; the remainder is more skewed to *RAS*, *PIK3CA* mutations and gene fusions than LCH (Figure 36.2). Clonal haematopoiesis may be a modifier that increases macrophage bias and explains the greater incidence in older males. Neoplastic macrophages infiltrate lipid-rich sites where interstitial macrophages are likely to be bone marrow-derived, producing many bizarre but characteristic pathologies.

ECD clinical presentation

ECD causes unique syndromes with highly specific features that should not be missed (Figure 36.5). About 90% of patients have long bone osteosclerosis that is painful in 38%. Appearances are often but not invariably dramatic and

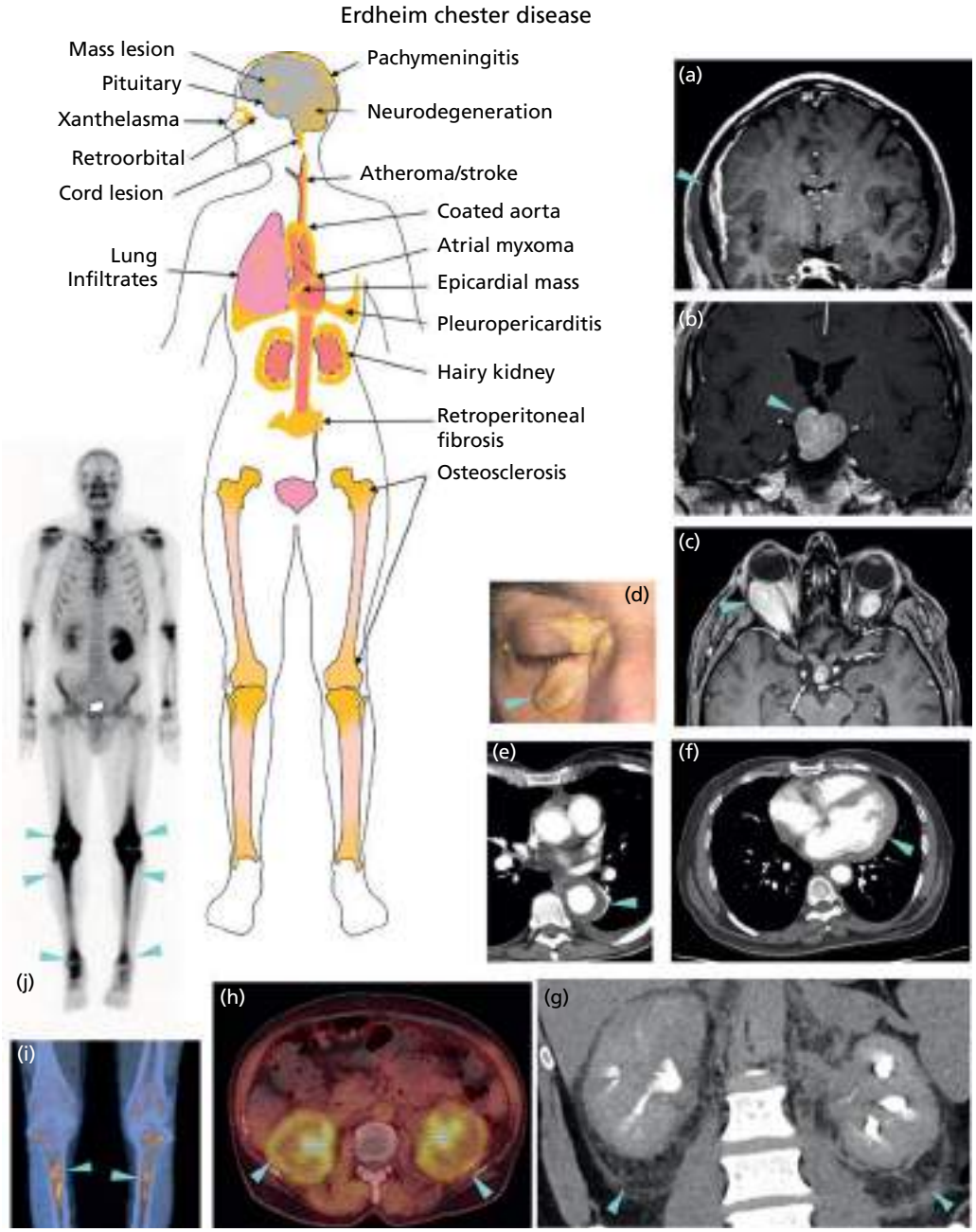

Figure 36.5 Clockwise from top right: (a) Pachymeningitis (dural thickening). (b) Suprachiasmatic mass. (c) Retro-orbital infiltration. (d) Xanthelasma. (Source: Courtesy of Ian Park). (e) Coated descending aorta. (f) Pericardial inflammation. (g) Perinephric inflammation (hairy kidney). (h) Hairy kidney PET-CT. (i) Long bone osteosclerosis PET-CT. (j) Long bone osteosclerosis bone scintigraphy.

symmetrical on PET-CT or bone scintigraphy. A minority of patients present with classic peri-orbital xanthomata. Inflammation of the peri-renal fat results in 'hairy kidney' on CT and retroperitoneal fibrosis presenting with obstructive nephropathy may cause irreversible ureteric obstruction. Aortitis may be diagnosed appearing as a 'coated aorta', a prominent sheathing of the adventitia, on CT. This is highly morbid causing stroke, coronary, renal and mesenteric ischaemia and infarction. The serosal surfaces may become thickened and inflamed causing pericardial and pleural effusions. Atrial myxoma, myocardial infiltration and epicardial inflammatory masses can lead to valvular obstruction, heart block and tamponade. Retro-orbital xanthoma with somatic mutation may trigger a diagnosis of ECD. In the CNS, diabetes insipidus is common, affecting about half of patients. Mass lesions may occur almost anywhere including the spine but dural thickening or pachymeningitis is a classic finding and may be confused with subdural haematoma. Scattered small lesions may occur in the cortex, periventricular white matter, brainstem, pons and cerebellum resulting in significant neurological deficits. Endocrine dysfunction and autoimmunity are common complications and patients frequently have fever and constitutional symptoms with leukocytosis, monocytosis and raised inflammatory markers. A staging system has not been agreed for ECD but it is clear that skeletal, cutaneous and peri-nephric infiltration are relatively low risk while cardiovascular and neurological involvement may be life-threatening. An urgent approach is also required to prevent loss of sight and irreversible ureteric obstruction.

ECD diagnosis

ECD is a clinical and radiological diagnosis supported by consistent pathology that excludes any other diagnosis. Recent series indicate an unacceptable mean delay of 2.7 years between the presentation and diagnosis of ECD, which should be a differential in a huge range of imaging abnormalities. Guidelines for diagnosis and management are now well described (see further reading). ECD should be considered in the differential of any adult presenting with xanthelasma, diabetes insipidus, bilateral osteosclerosis, retro-orbital inflammation, retroperitoneal mass or fibrosis, aortitis, pericarditis, atrial myxoma, atypical lung lesions, perinephric inflammation and unusual CNS lesions. A full blood work should document baseline leukocytosis, inflammatory markers and lipids toexclude cytopenias, autoimmunity, liver dysfunction and endocrine abnormalities. Immunoglobulins should be measured including IgG4 for potential overlap with Rosai Dorfman and IgG4 disease. Full body PET-CT should be performed to include the knees and ankles, MRI brain and gated cardiac CT or MRI are also essential for initial staging. High-resolution CT of the chest and dedicated vascular examinations may also be required. There should be a low threshold to examine the bone marrow and a myeloid NGS panel should be sent from blood or bone marrow to exclude myelodysplasia or myeloproliferative neoplasm. The best site for biopsy is superficial xanthelasmata which are accessible and cellular, followed by proximal tibia and perirenal biopsy although these are usually more fibrotic. Inflammatory masses in the retro-orbital space, dura, pericardium and other sites also provide very good material for genetics if the diagnosis is suspected. Sensitive PCR-based $BRAF^{V600E}$ testing should be performed on unfixed material, followed by DNA sequencing for other MAPK pathway mutations or deletions and RNA sequencing for gene fusion.

ECD management

ECD may respond to corticosteroids and chemotherapy but lesions are much less sensitive than LCH. Low-dose methotrexate and interferon alpha have supporting evidence. Anakinra, infliximab and tocilizumab may also be useful to suppress inflammatory cytokines but their use is not widespread. Sirolimus also is reported to have activity. Anything more advanced than cutaneous, skeletal and low-grade retroperitoneal and vascular disease should be treated with MAPK inhibitors. These can be life-saving in advanced cardiorespiratory compromise, vascular and CNS disease. Case series evidence supports vemurafenib or dabrafenib for *BRAF*-mutated disease and cobimetinib or trametinib for *BRAF* wild-type or uncharacterised genetics. Typically, 25–50% of the recommended dosing used in melanoma, effects rapid clinical improvement with tolerable side effects. Unlike melanoma, resistance is extremely rare and partial or intermittent dosing often circumvents toxicity. Dual agents are especially effective in $BRAF^{V600E}$ mutation and intermittent high dose pulses are recommended for achieving therapeutic levels in CNS disease. Similar to LCH, it has been difficult to withdraw treatment without rapid relapses. Neurological and psychological symptoms are reported by 92% of patients and specialist care is desirable. Five-year survival in specialist centres with access to inhibitors is 80%.

Juvenile Xanthogranuloma

The incidence of JXG is estimated at 1 per million children, about four-fold lower than LCH. It causes mostly solitary cutaneous lesions in infants, one-third presenting at birth and some in association with neurofibromatosis type 1. In fewer than 10% of cases, xanthomata may also occur in the liver, spleen, lung, soft tissues, eye, heart and CNS. The bone marrow may be infiltrated and there is a 20-fold increased risk of JMML. JXG is indistinguishable from ECD at the microscopic level, containing foamy macrophages with typical markers (CD68, CD14, CD163 and factor XIIIA) (Figure 36.6a). It has recently been shown that JXG is specifically associated with mutations in the receptor

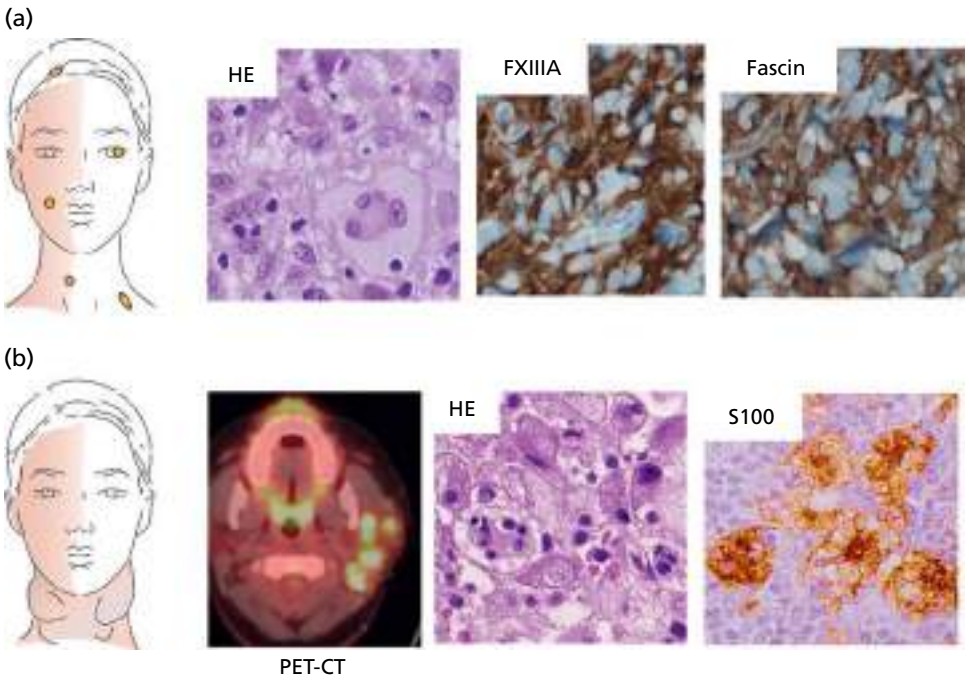

Figure 36.6 (a) The main features of JXG showing from left to right: superficial xanthogranulomata sometimes affecting the eye, foam cells and high expression of FXIIIA and fascin. (b) The main features of classical RDD showing from left to right: massive cervical lymphadenopathy and cells engaging in emperipopolesis (engulfment of viable cells) with S100 expression.

tyrosine kinase, *M-CSFR*. Lesions often spontaneously involute or respond to local therapy with surgery or cryotherapy. Systemic therapy may be required with prednisolone, vinblastine or methotrexate. Cytarabine and clofarbine have been used for more aggressive disease compromising the liver, trachea or CNS. MAPK inhibitors are likely to be highly efficacious but experience is limited.

Adult xanthogranulomas

Several rare macrophage-type histiocytic disorders are found in the C group (cutaneous) according to the Histiocyte Society classification. Somatic mutations have not been found yet.

Rosai Dorfman Destombes disease

Rosai Dorman Destombes Disease is a rare histiocytosis occurring in <1 per million population, first described by the French pathologist Destombes. The classic presentation, sinus histiocytosis with massive lymphadenopathy (SHML), describes very large painless cervical nodes that resolve spontaneously or with a short course of corticosteroids. This occurs mostly in children and young adults with a mean age of 21. About half of RDD cases are extranodal in subcutaneous tissue, exocrine glands, bone, orbits, pleural cavity, lungs and CNS. Sinus histiocytes have the phenotype of more recent monocyte derivatives and express S100 in addition to macrophage antigens CD68, CD163 (Figure 36.6b). They characteristically show emperipolesis or engulfment of other cells such as lymphocytes and granulocytes. RDD-like pathology may be reactive in SLE, JIA, IgG4 disease and lymphoma and *MAPK* mutations have only been reported in about half of cases (Chakraborty *et al*. 2021). RDD pathology is also seen in germline mutation of the *SLC29A3* gene which perturbs M-CSF receptor signalling and is manifest as Faisalabad histiocytosis and H syndrome. Single lesions rarely present clinically significant problems regardless of their true aetiology. Corticosteroids, vinca alkaloids, 6-mercaptopurine, methotrexate, cladribine, thalidomide and sirolimus have their advocates but radiotherapy has little efficacy. MEK inhibitors are effective when a mutation is discovered.

Malignant histiocytosis

Langerhans cell sarcoma and histiocytic sarcoma are acute histiocytic lesions with a high mitotic index and grave prognosis. They are probably best treated with radical local excision although recent reports suggest some may have targetable *MAPK* mutations. Follicular dendritic cell sarcoma is also high risk and difficult to cure. It is not haematological but mesenchymal in origin and benefits from a multi-disciplinary approach involving a sarcoma oncologist.

Hyperinflammation and haemophagocytic lymphohistiocytosis

Pathophysiology

In the steady state, tissue macrophages play a major role in maintaining immunological quiescence. A long-lived resident population engages in efferocytosis, the non-immune phagocytosis of dead cells, and a transient population of monocyte-derived cell patrols, probably leaving via the lymphatics. The full effector function of the macrophage is only unleashed when activated NK cells and T cells elaborate interferon-gamma (IFNγ). Originally described as 'Macrophage Activation Factor', IFNγ is the soluble mediator that effects immunity to viruses, mycobacteria, leishmania and other intracellular pathogens. Activated macrophages, in turn, promote adaptive immunity by releasing IL-1, IL-18 and IL-6. This feedback loop provides the ultimate cellular immune response against pathogens but is a potentially lethal force when unchecked (Figure 36.7). The grave illness caused by this phenomenon is described as Macrophage Activation Syndrome (MAS), corresponding to the pathological observation of haemophagocytic lymphohistiocytosis (HLH). More recently these have both been described as 'hyperinflammation'. HLH, the more familiar term in haematology, will be used here.

The HLH spectrum: primary and secondary

HLH is always multifactorial and attributable to an inflammatory trigger on a susceptible genetic background, sometimes compounded by acquired immune dysregulation. The two extremes of this spectrum have been characterised as 'primary' where inherited gene disorders cause a neonatal illness, triggered only by leaving the sterile environment of the uterus and 'secondary' where genetic susceptibility has not been identified but acquired immune dysregulation or potent inflammatory triggers are apparent (Figure 36.7). HLH becomes a risk in genetic or acquired loss of cytotoxic effector function because when cytotoxicity fails, pathogens and inflammatory triggers persist, driving a feed-forward loop of lympho-histiocytic activation and a state of hyperinflammation. The cytotoxic granule defects causing primary HLH are well defined and include severe phenotypes associated with mutation in *PRF1*, *UNC13D*, *STX11*, *STXBP2*, *RAB27A* or *LYST*. In general, less severe defects present later and older children and young adults display an increasingly diverse genetic background. In principle, almost any genetic primary immune disorder can predispose to HLH either through immunodeficiency and failure to deal with common viral triggers such as EBV and CMV or immune dysregulation manifest as auto-inflammation. EBV susceptibility in X-linked lymphoproliferative disorder (due to *SH2D1A* or *XIAP* mutation) and T cell defects in *ITK116*, *CD27* and *MAGT1* all predispose to HLH as do the lysosomal syndromes Griscelli and Chédiak–Higashi, and the inflammasome disorders arising from *CDC42* or *NLRC4* mutation.

Triggers of HLH are many and varied. Almost any virus, including COVID-19, is implicated. Herpesviruses EBV and CMV are common, mycobacteria, leishmania. Immune disorders associated with a significant risk of HLH are juvenile idiopathic arthritis and adult-onset Still's disease, Crohn's disease treated with azathioprine and haematopoietic stem cell transplantation. Malignancy is another significant trigger of HLH, especially lymphoproliferative disorders including PTLD, myelodysplasia, LCH and solid malignancy.

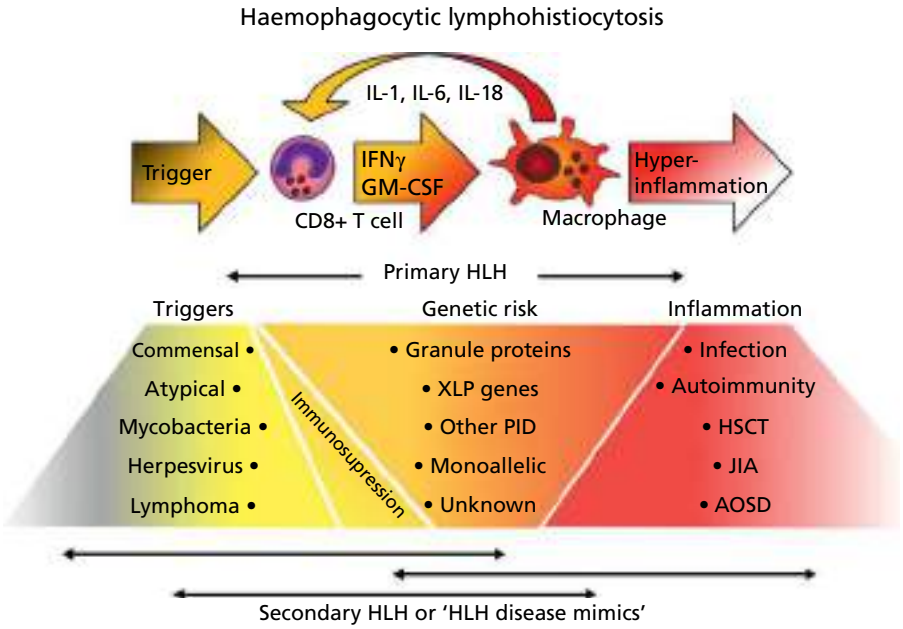

Figure 36.7 A schematic showing the main cellular circuit and risk factors predisposing to HLH. A given horizontal distance is required to reach a threshold of HLH, whether this is genetic in primary HLH or largely driven by acquired factors in secondary HLH syndromes, also described as 'HLH disease mimics'. (Source: Adapted from Jordan *et al.* 2019).

HLH epidemiology

The annual incidence of HLH is reported at about 1–2 per million, with the highest rate in infants <1 year of 10–20 per million. In the UK, the incidence has recently been estimated from electronic health records at 2 per million overall, with a rate of 14.6 per million in infants <1 year, decreasing to 0.8 in 15–44 year-olds and rising to 2.2 per million in >75 year-olds. These data also show much better one-year survival in children (77%) compared with older adults (30%).

HLH presentation and diagnosis

One of the most common misconceptions about diagnosing HLH is that it must be proven by the presence of haemophagocytosis on a bone marrow aspirate. This is only one criterion that may occur rapidly and subsequently vanish with the blood count. The classic triad of refractory fever, progressive cytopenia and organomegaly should prompt a rapid clinical and laboratory assessment for hyperinflammation. Two clinical scoring systems are in use: a paediatric score from the HLH2004 trial where >5/8 categorical variables trigger diagnosis and the HScore, a more sophisticated weighted score of nine staged variables (Table 36.2). The HScore was initially devised for adults but performs equally well in children and has the distinct advantage that all the parameters are routinely available, facilitating rapid diagnosis and monitoring. Both scores incorporate fever, organomegaly, cytopenia, fibrinogen, lipids, liver enzymes and ferritin. HLH 2004 criteria also include measurements of cytotoxic granule release and soluble CD25 (IL-2 receptor) but guidelines advise that pursuit of these results should not delay diagnosis. In adults, they play almost no role although the ratio of sCD25:ferritin may be useful for identifying lymphoma-associated HLH. The HScore is also marginally superior to the HLH 2004 criteria in critically ill patients. Important clinical features that influence the course of the illness include cytopenia, sepsis, liver failure and neurological dysfunction. Isolated CNS HLH is particularly challenging. A multi-disciplinary approach is beneficial, especially in adults who may have a range of comorbidities and potential triggers.

Management of primary HLH in infants

HLH in neonates and infants has a strong genetic component, often already recognised as familial and rapidly ascertainable by germline DNA sequencing in de novo cases. Infection and malignancy should also be rigorously excluded by viral PCR, cultures, PET-CT and bone marrow examination. Immunosuppressive treatment is urgently required, often as a bridge to allogeneic stem cell transplantation in severe phenotypes associated with cytotoxic granule defects. The HLH 2004 protocol is based on induction with dexamethasone, IVIg, ciclosporin and pulsed etoposide. Intrathecal treatment is given for CNS disease. Although this approach was life-saving when first introduced, the

Table 36.2 Comparison of HLH criteria.

		HSCORE	**>169 = 90% accurate**	
HLH 2004	**5/8**	**Immmunosuppression**	**18**	
Fever	1	Fever: >38.4, >39.4	33	49
Splenomegaly	1	Organomegaly: one, two	23	38
Cytopenia: two	1	Cytopenia: two, three	24	34
Hb <90		Hb <92		
Plt <100		Plt <110		
Neut <1		WBC <5		
Ferritin ≥ 500 µg/L	1	Ferritin>2000, >6000 µg/L	44	64
Triglycerides ≥265 mg/dL *or* Fibrinogen ≤1.5 g/L	1	Triglycerides>1.5, >4.0 mg/dL	44	64
		Fibrinogen >2.5 g/L	30	
		AST >30 U/mL	19	
Haemophagocytosis		Haemophagocytosis	35	
NK cell function	1			
sIL-2R ≥ 2400 U/mL	1			

mortality of primary HLH has not improved in the last decade and newer cytokine-based approaches are evolving, including emapalumab (IFNγ), tocilizumab (IL-6) anakinra (IL-1) and JAK inhibition. Cytokine-based approaches are proven to be effective but universal access is currently ensnared by regulation. ATG or alemtuzumab are also useful as a preconditioning step to achieve transplantation in refractory patients. Allogeneic transplantation is indicated for mutation in *PRF1*, *UNC13D*, *STX11*, *STXBP2*, *RAB27A*, *LYST* or *SH2D1A* but is not straightforward since reduced intensity conditioning is associated with partial chimerism and graft failure.

Management of HLH in older children and adults

Significant triggers are required to initiate HLH in older children and adults. Prompt investigation of viral infection or reactivation, bacterial infection, other pathogens, autoimmunity and lymphoproliferative disorders should be initiated with viral PCR, cultures, autoantibody titres, PET-CT and bone marrow examination. Flow cytometry of blood bone marrow and even splenic aspirate maybe required to detect intravascular or splenic lymphoma. MRI brain and CSF examination are indicated with even mild neurological impairment. EBV and CMV should be treated with rituximab and ganciclovir, respectively. Broad-spectrum antibiotics, antifungals and even empiric mycobacterial treatment are warranted, pending culture results. Successful treatment will often resolve HLH eventually but parallel suppression of hyperinflammation should not be delayed. While the HLH 2004 approach is tolerated and widely used in older children, it often fails in adults reported. A dismal median survival of 3.2 months and overall survival of 35% have been reported partly because highly immunosuppressive and cytoreductive therapy are hazardous in the face of serious infection and mask the diagnosis of lymphoma, a major trigger. Important lessons have been learned from the use of anakinra by rheumatologists treating MAS in patients with autoimmune disease. It is possible to avoid etoposide and to minimise steroid exposure in most patients. Suppression of IFNγ with emapalumab, IL-6 with tocilizumab and JAK-STAT signalling with ruxolitinib or baricitinib are also proving successful in early phase studies. As in primary HLH, alemtuzumab is a potent salvage agent but commits the patient to allogeneic transplantation.

Conclusion

The macrophage and DC systems have unique developmental pathways and roles in host defence that set them apart from other components of the human haematological system. Deficiency, neoplasm and hyperactivation lead to a wide spectrum of pathology formerly known as 'histiocytosis'. New developments in molecular pathology are having profound effects on diagnosis and management.

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CHAPTER 37

Stem cell transplantation

37

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Introduction

The first haematopoietic stem cell transplants were performed in 1957. Although clinical outcomes were dismal initially, the vision of clinical pioneers, such Don Thomas, led to the demonstration more than a decade later that transplantation of bone marrow from HLA-matched siblings had the capacity to deliver long-term disease-free survival in a small proportion of patients with refractory leukaemia. Despite continued widespread scepticism, slow but steady progress continued to be made in the development of more effective forms of graft-versus-host-disease prophylaxis; coupled with advances in supportive care, the immediate toxicity and long-term morbidity of transplantation significantly reduced during the 1970s. At about the same time, transplantation of cryopreserved autologous bone marrow was shown to be a curative procedure in a proportion of patients with relapsed Hodgkin lymphoma. Although allogeneic transplantation was initially limited to patients with an HLA-matched sibling donor, the pioneering work of the Anthony Nolan and other nascent donor registries across the world demonstrated that it was possible to use HLA-matched unrelated donors as an alternative stem cell source in patients lacking a suitable sibling donor. More recently, the advent of reduced intensity conditioning regimens coupled with the growth of unrelated donor registries worldwide and the successful utilisation of cord blood cells and, more recently, haploidentical donors has dramatically increased access to transplant for patients with haematological malignancies, bone marrow failure and haemoglobinopathies.

Immunological basis of stem cell transplantation

The major complications of allogeneic SCT are caused by the immunological responses triggered by the infusion of donor haemopoietic progenitors and lymphocytes into an immunosuppressed host. These can take the form of either a host-versus-graft (HVG) or a donor-derived graft-versus-host (GVH) response. Clinically, the HVG response can result in graft rejection, while a GVH response may manifest itself as either graft-versus-host disease (GVHD) or a graft-versus-leukaemia (GVL) reaction (Chakraverty and Teshima 2021). It is now possible to blunt the HVG reaction by optimising the immunosuppressive properties of the conditioning regimen and consequently, graft rejection is rare in most clinical settings. In contrast, GVHD and disease relapse remain the major complications of allogeneic stem cell transplantation, and novel approaches that permit the induction of a GVL effect without inducing GVHD-related host injury are required. Conversely, autologous SCT is a relatively unremarkable immunological event in which such allogeneic responses are absent.

The human leucocyte antigen system

The human leucocyte antigen (*HLA*) genes are located within the major histocompatibility complex (MHC) on the short arm of chromosome 6 (6p21.3) (Figure 37.1) (Cornaby *et al.* 2021). *HLA* class I genes encode polypeptides expressed as cell-surface

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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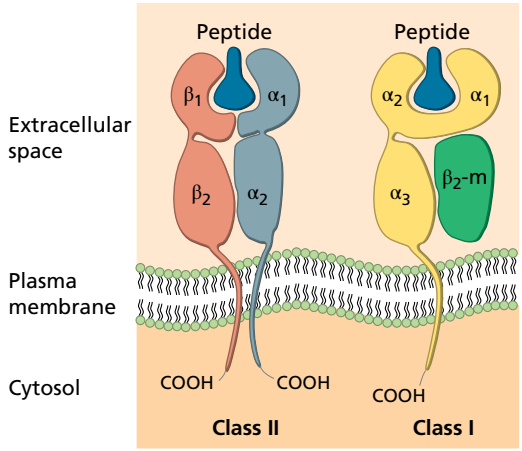


Figure 37.1 Diagrammatic representation of HLA class I and II molecules showing protein domains and bound peptide.

transmembrane glycoproteins in complex with a soluble protein, β_2-microglobulin. The three classical class I proteins, HLA-A, HLA-B and HLA-C are expressed on virtually all nucleated cells and also on platelets. HLA class II molecules possess a similar structure to HLA class I molecules and are composed of two MHC-encoded polypeptide chains (α and β). The three HLA class II molecules, HLA-DR, HLA-DQ and HLA-DP, have a restricted tissue distribution; they are mainly expressed on antigen-presenting cells such as B cells, dendritic cells and macrophages, but can be expressed on other cells in the context of inflammation.

Both class I and II HLA molecules share a similar structure of four extra-membranous domains. The two most membrane-distal domains of HLA class I (α1 and α2) and HLA class II (α1 and β1) contain a cleft available for binding of small peptides. Thus, both class I and class II proteins can be considered trimolecular proteins consisting of three subunits: HLA heavy chain, β_2 -microglobulin and peptide form class I molecules, and HLA α-and β-chains and peptide form class II molecules. The binding of peptides is key to the functions of the classical HLA molecules. The source of peptides and the binding procedure differ for the two classes of molecules. HLA class I molecules are assembled within the endoplasmic reticulum (ER), where chaperone proteins mediate the association with β_2 -microglobulin and peptide. Peptides are derived from endogenous molecules (e.g. self or viral proteins) by cytosolic degradation within the proteasome, a multicatalytic protein complex, before being actively transported into the ER. Once the HLA class I trimolecular complex is formed, the molecule leaves the ER and traffics to the cell surface via the Golgi apparatus. The formation of a peptide-bound HLA class II molecule is distinct in several ways. Both α-and β-chains are directed to the ER, where they first associate with the transmembrane invariant chain. The invariant chain blocks binding of ER-resident peptides and instead directs the class II molecule via the Golgi apparatus to endosomal vesicles. Peptides contained in the endosome are derived from the processing of exogenous proteins (e.g. derived from microbes or dead cells). Within the endosome, the invariant chain is cleaved in a series of steps and specific peptide binding is catalysed by HLA-DM before the HLA class II–peptide complex is transported to the cell surface.

T cells and natural killer (NK) cells both possess receptors that recognise and interact with HLA molecules (see also Chapter 4). T cells expressing the CD4 molecule recognise HLA class II molecules and T cells expressing CD8 recognise HLA class I molecules. The specificity of interaction is determined during development of T cells within the thymus, such that circulating T cells should not interact with HLA molecules presenting peptides derived from normally expressed 'self' proteins. However, the presentation of 'non-self' peptides, for example peptides derived from viral or bacterial proteins initiate activation signals mediated by the T-cell receptor and coreceptors, resulting in the generation of an immune response against the cells expressing the target HLA protein–peptide complex. NK cells express a highly diverse repertoire of receptors, including killer-cell immunoglobulin-like receptors (KIRs) that elicit either activation or inhibitory signals. Interaction between an NK cell and a target cell is influenced by the interaction between certain HLA class I molecules on the target cell (e.g. HLA-B and C) and KIRs on the NK cell. Interactions between an HLA class I molecule and a corresponding KIR may result in a negative signal that outweighs any activation signals; thus, the NK cell will not attack the target cell. However, if a cell has lost expression of HLA molecules as a result of malignancy or viral infection, the absence of the inhibitory signal may result in activation signals being dominant and allow NK cell-mediated attack on the target cell.

The outstanding feature of the *HLA* genes is their extensive polymorphism (www.ebi.ac.uk/imgt/hla/). At each of the genes, there are multiple possible variant alleles and the majority of this variation occurs within regions encoding the peptide binding domains. Because different HLA proteins bind peptides with different sequences, one of the functions of *HLA* polymorphism is to allow the presentation of numerous different peptides to the immune system. As there are six antigen-presenting 'classical' HLA molecules (HLA-A, -B, -C, -DR, -DQ, -DP) and most individuals are heterozygous for these loci, potentially each individual has 12 different HLA molecules, each of which can bind thousands of different peptides. Evolution of *HLA* polymorphism occurred as a result of selective pressure imposed by geography, climate and infectious pathogens so that the repertoire of HLA alleles varies between different populations.

Detection of HLA polymorphisms is performed using DNA methods, including PCR with sequence-specific primers or sequence-specific oligonucleotide probes; however, these approaches are being rapidly superseded by high-throughput, low-cost automatic next-generation sequencing technologies.

Each HLA allele is given a unique numerical designation. For example, for the allele *HLA-A* 02:101:01:02N,* the gene name *HLA-A* is followed by an asterisk (*) and then a series of four fields separated by colons (:). The first field indicates the allele group (02), which often corresponds to the broad serological antigen encoded by the allele. The second field (101) indicates the subtype, a number assigned in the order in which the DNA sequences were determined. Alleles whose numbers differ in the first two fields must differ in one or more nucleotide substitutions that change the amino acid sequence of the encoded protein. The third field is used to name alleles that differ only by synonymous nucleotide substitutions (also called silent or non-coding). The fourth field is used to name alleles that differ in either intron or 3′ or 5′ regions of the gene. Lastly, an allele may have a suffix indicating aberrant expression; for example *N* indicates that it is a null allele with no protein being expressed, *L* indicates low cell-surface expression and *S* indicates that the molecule is expressed only in a soluble form.

HLA matching for transplantation

The frequency of T cells capable of reacting against a foreign antigen is of the order of 1 in 10–100,000. However, if cells from two *HLA* disparate individuals are mixed, the frequency of responding (or 'alloreactive') cells can be as high as 1–10%. Excessive alloreactivity occurring after allogeneic SCT can result in graft failure or GVHD. To reduce alloreactivity following allogeneic SCT, it is necessary to define the *HLA* type of donor and recipient. The degree of *HLA* matching informs not only the choice of donor, but also the selection of conditioning protocol and format for GVHD prevention.

In situations where the donor is genetically identical to the recipient (syngeneic transplantation), graft rejection or significant GVHD are not observed. For patients undergoing allogeneic transplantation using an HLA-identical sibling donor, there is still a significant risk of GVHD as a result of polymorphisms outside the HLA system; these generate so-called 'minor' histocompatibility antigens that are presented as processed peptides in the context of 'self' HLA molecules. For any individual donor and recipient pair, there will be several thousand non-synonymous mismatches in coding SNPs, although only a minority will generate peptides that can bind to HLA with high affinity. Although naïve T cells reactive to mismatched minor antigens can induce GVHD, previous exposure to minor antigens may increase this risk. For example, male patients will possess antigens encoded by Y chromosome genes that are absent in females (HY antigens). Female donors, specifically multiparous individuals who have had male infants, may have been primed against HY antigens and as a consequence have circulating memory T cells that recognise male patient cells expressing these proteins. This is likely to underlie the increased risk of GVHD in male recipients of female grafts.

The chances of a sibling being HLA-identical to a patient is theoretically one in four and, taking into account the size of most families, about a third of patients will have an HLA-identical sibling donor. Another source of HLA-matched donors is the various volunteer donor registries (such as the Antony Nolan Registry and the US National Marrow Donor Program) that exist throughout the world and that have accrued over 40 million donors (Karpova *et al.* 2019). The likelihood of finding an optimal donor matched for *HLA-A, HLA-B*, *HLA-C* and *HLA-DRB1* alleles is about 75% amongst whites of European descent, but significantly lower for other ethnic groups. Although single HLA mismatches can be reasonably well tolerated clinically, greater degrees of mismatch are associated with increased rates of graft rejection or GVHD. Certain *HLA* mismatches may be 'permissive' so that they do not result in a worse transplant outcome. For example, a subset of permissive mismatches at *HLA-DPB1* (a locus not usually considered in matching with unrelated donors) may actually be associated with an improved outcome because of a lower risk of disease relapse, although this is not a consistent finding across all studies. Newer transplant strategies (e.g. use of post-transplant cyclophosphamide [PTCy] or abatacept) may permit unrelated donor grafts with even greater degrees of *HLA*-mismatch to be tolerated.

Additional options include *HLA*-mismatched related (usually haplo-identical) or umbilical cord blood donors. Cord blood maybe a useful option because recipients tolerate a greater degree of *HLA* mismatching than is possible with adult grafts, most likely reflecting the reduced alloreactivity of fetal T cells. Cord blood units mismatched for one or two HLA loci are available for the majority (>80%) of adult patients. The main limiting factor is the relative lack of cord blood donations with sufficient numbers of HSCs to ensure engraftment, especially for larger adults. This deficiency could be overcome by using dual cord transplants, where ultimately long-lived haematopoiesis is derived from one of the cord units. Although comparative trial data do not yet suggest an advantage over cord blood, related haplo-identical donors are increasingly used based upon updated GVHD prevention and ease of procurement. *In vivo* purging of alloreactive T cells using PTCy, or alternatively using methods to completely deplete T cells from the graft are both effective at reducing the risk of GVHD. In some situations, a haplo-identical donor may be chosen over a single HLA antigen-mismatched unrelated donor, for example under conditions where the former donor can offer greater protection against CMV infection. Where HLA-mismatched donors are used, it is important to screen recipients for antibodies that react to mismatched donor HLA antigens, as this can be linked to a higher risk of graft rejection. For both cord and haploidentical SCT, there is growing interest in other factors that influence outcomes. For example, the risks of transplantation may also be influenced by the complex interaction between mismatching at HLA class

I and the repertoire of activating or inhibitory *KIR* genes inherited by the donor. Furthermore, there is also evidence that recipients of cord or haploidentical grafts may be less prone to develop GVHD if they possess a haplotype that is absent in the donor, but is shared with the donor's mother; in this scenario, *in utero* exposure to non-inherited maternal antigens (NIMAs) is postulated to induce tolerance of donor T cells.

Acute graft-versus-host disease

Acute GVHD is a complex immunological disorder in which donor T cells initiate tissue damage consequent upon recognition of recipient antigens not expressed in the donor. The recipient is unable to reject the donor T cells because of the immunosuppressive effects of conditioning therapy. Chemotherapy-or radiation-induced tissue damage leads to the release of proinflammatory cytokines and altered chemokine or adhesion molecule expression. These changes impact on the developing alloreactive T-cell response, leading to further cytokine dysregulation and enhanced trafficking of cellular effectors to the organs targeted in GVHD.

Initiation of GVHD (Figure 37.2) requires presentation of host antigens to donor T cells, although there appears to be considerable redundancy in the type of antigen-presenting cells involved. In pre-clinical models, $CD8^+$ T cells and $CD4^+$ T cells differ in terms of whether they require priming by professional antigen-presenting cells; in the latter case, upregulation of HLA Class II in inflamed peripheral tissue cells, for example in the gut, may be sufficient to trigger a response. Conditioning-related tissue injury leads to the release of damage-associated molecular patterns (DAMPs, e.g. uric acid and ATP) or exposure to pathogen-associated molecular patterns (PAMPs, resulting from entry of microbes across damaged epithelia). Recognition of DAMPs or PAMPs by cell-surface or intracellular pattern-recognition receptors induces the release of proinflammatory cytokines, such as interleukin (IL)-1β. Enhanced expression of HLA and costimulatory (e.g. B-7 family) molecules upon host cells promotes their priming capacity. Primed donor T cells rapidly undergo proliferation and develop a multitude of effector functions, including the capacity to generate proinflammatory cytokines (e.g. TNF-α, interferon-γ and IL-17) or the upregulation of cytotoxic machinery (e.g. perforin/granzyme B). The primed effector T cells then traffic in large numbers to epithelial target cells and recruit other cellular effectors such as neutrophils, macrophages and NK cells, which in concert cause further damage.

Epidermal stem cells in the skin and crypt-associated stem cells in the gut are particularly sensitive to immune injury; the consequent loss of epithelial regenerative capacity at these sites exacerbates disease. In parallel, damage to specialised intestinal epithelial cells, for example Paneth cells that produce anti-microbial peptides, can lead to major shifts in the overall composition and diversity of the gut microbiome. In a feed-forward loop, overgrowth of particular pathogenic bacterial species can lead to further loss of epithelial integrity, for example by reducing the concentrations of microbial metabolites that have a gut-protective functions. Other factors such as use of broad-spectrum antibiotics or reduced enteral nutrition may also impact on these processes and increase the risk of gut injury.

Chronic graft-versus-host disease

Chronic GVHD is a disorder associated with continuing host injury, profound immunodeficiency and autoimmunity. One of the key features is fibrosis which is a consequence of maladaptive repair following initial acute tissue injury. For example, in the skin and lung, pro-inflammatory Th17 cells induce recruitment of monocytes that differentiate into pathogenic macrophages producing TGF-β, which in turn drives local myoblast differentiation. Myofibroblasts deposit excess collagen that drives organ dysfunction (e.g. bronchiolitis obliterans, sclerodematous-like skin changes). The thymic stroma is also a target of GVHD; this may lead to reduced naive T cell output as well as impaired negative selection of autoreactive T cells which have the potential to damage peripheral tissues. Damage to lymph node stroma may also impair T-cell homeostasis and disrupt peripheral tolerance mechanisms (e.g. reducing Treg survival or function, or failure to delete autoreactive T cells). These findings may help to explain why individuals with chronic GVHD develop simultaneous features of immunodeficiency (infections) and autoimmunity (e.g. immune cytopenias, sclerodermatous change). Host-reactive $CD4^+$ T cells (e.g. follicular helper T cells) might provide helper functions for B cells leading to the production of allo- or auto-antibodies which may provoke macrophage activation and further reinforce the fibrotic process. Accordingly, some patients with chronic GVHD may respond to anti-B-cell monoclonal antibody therapy or imatinib.

Graft-versus-leukaemia effect

The importance of an immunologically mediated GVL effect in contributing to the curative effect of allogeneic SCT is supported by the observations that T-cell depletion (TCD) increases the risk of relapse and that patients who develop GVHD have a lower risk of disease relapse. Conclusive evidence was provided by the demonstration that infusion of donor lymphocytes can produce durable remissions in patients who have relapsed after allogeneic SCT. It has subsequently been shown that donor lymphocyte infusion (DLI) is a remarkably effective salvage therapy in chronic myeloid leukaemia (CML), with more than 80% of patients achieving a sustained molecular remission. Durable responses can also be achieved in patients transplanted for indolent lymphoma and Hodgkin lymphoma, with responses

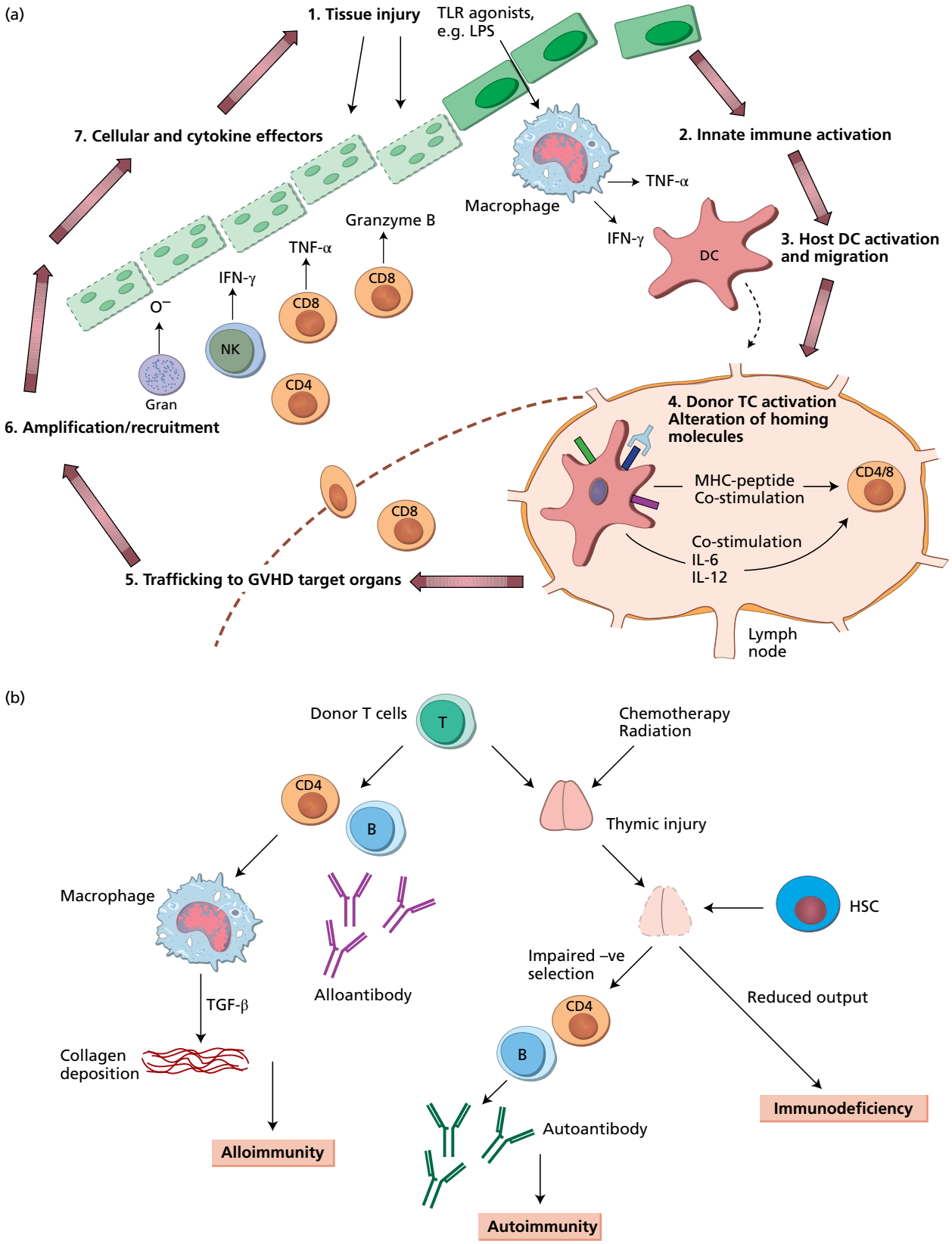


Figure 37.2 Pathophysiology of acute GVHD (a) and chronic GVHD (b). See text for explanation of abbreviations.

being observed less commonly in patients with aggressive lymphoma, myeloma or acute leukaemias. As would be predicted, DLI can be complicated by the development of GVHD, although the risk of this life-threatening complication is reduced if the lymphocytes are infused following a significant delay (>12 months) after transplantation or by using an escalating dose schedule rather than a single 'bulk' infusion. For the most part, the antigens recognised in a GVL reaction overlap with those recognised during GVHD. Clinical separation of GVL and GVHD may reflect increased sensitivity of normal or malignant haemopoietic tissue to an emerging GVH reaction compared with epithelial cells. Selective expression of antigenic targets of a GVH reaction on haemopoietic tissues (e.g. the minor H antigen HA-1, proteinase 3 or WT1) or leukaemic blasts (e.g. the product of the mutated *NPM1* gene in AML) may also underlie the development of a GVL reaction in the absence of GVHD. This concept forms the basis of novel strategies to deliver a GVL effect without a concomitant risk of GVHD, such as peptide vaccination, or gene transfer of T-cell receptors (TCRs) or chimeric antigen receptors (CARs) specific for leukaemic antigens.

Immune reconstitution

Allogeneic SCT is followed by a prolonged period of cellular and humoral immunodeficiency, while donor-derived immune recovery occurs. Reconstitution of an immune response after transplantation can be evaluated in the clinic by monitoring the absolute numbers of T (CD4, CD8), B and NK cells. NK cell numbers recover most rapidly while other subsets (especially $CD4^+$ T cells and B cells) recover more slowly. In patients who have received a TCD graft, T-cell recovery is substantially delayed. Analysis of thymic function after transplantation can be evaluated by measuring the episomal DNA excision circles of the TCR δ locus deleted during recombination of the TCR in functional αβ T cells (known as TREC, for T-cell receptor excision DNA circles). TREC levels are low for the first 6 months following transplantation and recover thereafter. As a consequence, the recovering T-cell repertoire is initially limited and mostly dependent on peripheral expansion of donor memory T cells. Thymic function is reduced in adults and may be further compromised by the effects of chemoradiotherapy and GVHD. Significant HLA mismatching between donor and recipient may also lead to 'holes' within the T-cell repertoire due to perturbations in thymic selection. These defects reflect a failure of donor-derived thymic emigrants to interact with peptides presented in the context of 'foreign' host HLA molecules. Quantitative B-cell deficiency is present in virtually all patients in the first months after transplantation and may persist for a number of years post-transplantation as a consequence of reductions in the number of marrow B-cell precursors, particularly in patients with chronic GVHD. This defect in B-cell production has multifactorial causes, including damage to the bone marrow stroma, the deleterious effect of inflammatory cytokines and the lymphocytic effects of glucocorticoid therapy. The source of stem cells also influences the kinetics of immune reconstitution. Thus, peripheral blood stem cell (PBSC) grafts, which contain higher numbers of haemopoietic progenitors and mature T cells, are associated with more rapid immune reconstitution than bone marrow grafts. Cord blood transplantation may be affected by poor immune reconstitution in adults since the number of haemopoietic progenitors is often limited and the transferred T cells are naive. Thus, patients are at heightened risk of re-activation of cytomegalovirus (CMV) or Epstein–Barr virus (EBV).

Stem cell mobilisation

Biology of stem cell mobilisation

The ability of both cytokines and chemotherapy to mobilise HSC from the bone marrow into the peripheral blood has transformed clinical practice such that mobilised peripheral blood stem cells (PBSC) are now the most widely used stem cell source in both autograft and allograft recipients. The localisation of haemopoietic stem and progenitor cells within the bone marrow cavity is mediated through the binding of a range of adhesion molecules, including CXCR4 and VLA4, with their cognate ligands on bone marrow stroma. Administration of haemopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) disrupts the adhesion of progenitors to the bone marrow stroma, resulting in their mobilisation into the peripheral blood in large numbers. Antagonists to CXCR4, such as plerixafor, significantly augment G-CSF-mediated stem cell mobilisation and this has transformed mobilisation options in candidates for an autologous transplant in whom low levels of stem cell mobilisation can be predicted. Importantly there is also now substantial interest in the development of novel stem cell mobilisation strategies in order to optimise the delivery of genetically manipulated HPC (Karpova *et al.* 2019).

Stem cell mobilisation in clinical practice

In patients undergoing autologous SCT, administration of G-CSF as monotherapy represents the commonest method of stem cell mobilisation although G-CSF in conjunction with salvage chemotherapy remains an important mobilisation strategy in certain patient subgroups. G-CSF can be administered as an outpatient and, in one commonly used schedule, patients receive four daily subcutaneous injections of G-CSF, with stem cell harvesting scheduled for day 5 and 6. G-CSF at a dose of 5–10 μg/kg/day is typically reasonably well tolerated although commonly associated with myalgia, bone pain and headache which are usually easily managed with symptomatic therapy including paracetamol. Splenic rupture has been reported as a very rare

occurrence in patients undergoing G-CSF-stimulated HPC mobilisation and it is wise to advise patients to report any unexpected abdominal or pleuritic pain during the period of G-CSF administration. Using G-CSF-based mobilisation strategies, a minimum target cell dose of 2×10^6 $CD34^+$ cells/kg is achieved in more than 80% of patients. In patients who fail to mobilise the target number of $CD34^+$ cells, the CXCR4 antagonist plerixafor can be successfully combined with G-CSF and this advance now permits mobilisation of the required numbers of PBSC in more than 95% of autograft candidates. Initially used in patients who had previously failed to mobilise in response to G-CSF alone or G-CSF/chemotherapy, plerixafor can now be pre-emptively administered in patients undergoing G-CSF mobilisation who can be predicted to be poor mobilisers on the basis of low numbers of circulating $CD34^+$ cells in the peripheral blood on the first day of leukapheresis (Figure 37.3). In the small minority of eligible patients with relapsed lymphoma who fail to mobilise adequate numbers of PBSCs using combined G-CSF/plerixafor administration, alternative consolidation strategies such as allogeneic transplantation should be considered

G-CSF-mobilised PBSCs also represent the commonest stem cell source in patients undergoing allogeneic SCT. The apparent safety of G-CSF doses in the range routinely used for autologous stem cell mobilisation (10–15 μg/kg for 4–6 days) has resulted in its adoption as the standard approach for stem cell mobilisation in sibling and, increasingly, adult unrelated donors. The options of both PBSC donation and bone marrow harvest, as well as any expressed preference of the transplant centre, are discussed with volunteer unrelated donors. Donors who elect to receive G-CSF also need to be aware of the 2–3% chance that an additional bone marrow harvest will be required if G-CSF fails to mobilise sufficient PBSC. Initial concerns that G-CSF may predispose to the subsequent development of secondary haematological malignancies in normal donors have not been substantiated. The effects of G-CSF on the fetus are not completely understood and all female donors of childbearing age should have a negative pregnancy test prior to its administration.

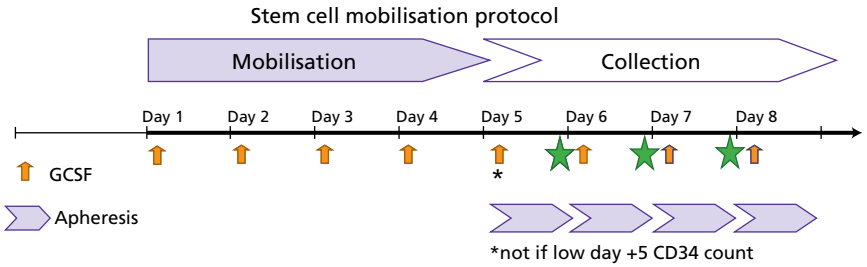
Choice of stem cell source and dose

Autologous transplantation

In autologous transplant recipients, the use of PBSC as a stem cell source is associated with accelerated neutrophil and platelet engraftment compared with those observed using harvested bone marrow; coupled with their ease of procurement, this means that PBSC are almost universally used of as the stem cell source in autograft recipients. The minimum stem cell dose in autologous transplants is 2×10^6 $CD34^+$ cells/kg. Although increasing the number of $CD34^+$ cells transplanted hastens neutrophil and platelet engraftment, there is little evidence that transplantation of more than 5×10^6 $CD34^+$ cells/kg is beneficial and there remain concerns, largely unsubstantiated, that higher stem cell doses may be associated with an increased risk of tumour contamination. In patients transplanted for myeloma, many centres aim to harvest a minimum of 4×10^6 CD34-positive cells/kg, using G-CSF alone or in combination with plerixafor, in order that sufficient cells are available for the option of a tandem transplant, or second autograft at the time of relapse.

Sibling and unrelated donor transplantation

PBSCs are the most frequently used stem cell source for patients undergoing a sibling allograft and are increasingly used in unrelated donor transplant recipients. Transplantation of PBSCs results in earlier neutrophil and platelet engraftment, which may reduce transplant-related mortality (TRM), particularly in patients with advanced leukaemia. In patients transplanted using a reduced intensity conditioning (RIC) regimen, PBSC are the preferred stem cell source. In patients transplanted using a myeloablative conditioning regimen (MAC), there are randomised



Day	1	2	3	4	5	6	7	8
G-CSF	✓	✓	✓	✓	✓	✓[a]	✓[a]	✓[a]
Plerixafor injection [0.24 mg/kg] (only if low circulating CD34 count on day +5)					✓[a]	✓[a]	✓[a]	
Apheresis*					✓[a]	✓[a]	✓[a]	✓[a]

[a] If necessary

Figure 37.3 'Pre-emptive' mobilisation schedule.

data demonstrating equivalence of survival in patients undergoing transplantation from a matched unrelated donor transplanted using PBSC or bone marrow as the stem cell source. There were no differences in the rates of acute GVHD or disease relapse. Of note, however, while the rates of primary graft failure in patients transplanted using PBSC were lower, more chronic GVHD was reported although whether these observations apply in patients receiving TCD as GVHD prophylaxis is unclear. Despite these observations, and perhaps because of the more widespread adoption of *in vivo* TCD in GVHD prophylaxis, the use of PBSC as the preferred stem cell source in patients transplanted for haematological malignancies continues to increase. Importantly, in patients transplanted for aplastic anaemia, where prevention of chronic GVHD is a key consideration, and in paediatric transplants, bone marrow remains the preferred stem cell source in many centres.

Cell dose is an important factor determining outcome after both matched sibling and unrelated donor transplants, likely consequent on accelerated immune reconstitution in recipients of a higher stem cell dose. The lowest acceptable stem cell dose to secure engraftment in recipients of a PBSC allograft is considered to be 2×10^6 CD34-positive cells/kg (2×10^8 mononuclear cells/kg if bone marrow is being used), although in practice the great majority of patients transplanted with a PBSC graft containing in excess of 1×10^6 CD34-positive cells/kg will still engraft. Since outcome is improved with higher doses of CD34-positive cells, most centres aim for a dose in the region of 4×10^6 CD34-positive cells/kg. In patients transplanted from an HLA-identical sibling using a T-replete myeloablative regimen, there is some evidence that the incidence of chronic GVHD is increased if the stem cell dose exceeds 8×10^6 CD34-positive cells/kg. In this setting, it may therefore be reasonable to have a target dose of $4–8\times10^6$ CD34-positive cells/kg. However, there is no evidence, as yet, that there should be an upper limit on cell dose in patients transplanted using an RIC regimen or if the graft is T-cell depleted.

Haploidentical stem cell transplantation

The last decade has seen a dramatic increase in the number of children and adults transplanted using a haploidentical donor. Historically, the degree of patient-donor HLA disparity associated with the use of a haploidentical donor resulted in prohibitive rates of graft failure and severe GVHD. Initial approaches used stringent *ex vivo* TCD coupled with an intensely immunosuppressive conditioning regimen and transplantation of high 'mega' doses of CD34-positive cells to overcome these barriers. While encouraging results were reported from selected centres, an unacceptably high incidence of viral infections and complex post-transplant morbidities prevented this approach being widely adopted. More recently, two approaches have proved effective at reducing the risk of graft failure and acute GVHD. The administration of PTCy, which results in the *in vivo* depletion or anergy of alloreactive host and donor T cells in patients transplanted using an unmanipulated bone marrow or PBSC results in high rates of neutrophil and platelet engraftment while simultaneously reducing the risk of severe acute and chronic GVHD in recipients of transplants from both mismatched unrelated and haploidentical donor. Although the extent to which PTCy abrogates a GVL effect remains uncertain, particularly in recipients of a RIC regimen, this innovative GVHD prophylaxis strategy has transformed the deliverability of haplo-identical transplants and resulted in a rapid increase in the number of such procedures delivered worldwide (Fuchs *et al*. 2021). Importantly, the PTCy platform also appears a very promising GVHD prophylaxis strategy in recipients of mismatched unrelated donor transplants potentially increasing transplant accessibility for patients from ethnic minorities (Shaw *et al*. 2021). An alternative approach, which yields particularly encouraging results in children, uses *ex vivo* CD3+/CD19+ or alternatively αβ TCD and represents an important alternative approach to haploidentical transplantation, particularly in the paediatric setting.

Cord blood transplantation

UCB remains a valuable stem cell source in paediatric and adult transplantation. Underpinning its importance is the observation that comparable degrees of HLA disparity result in lower rates of severe GVHD after a cord blood transplant than would be expected using a mismatched unrelated donor. This has important implications in terms of increasing donor options in patients with uncommon HLA types for whom a suitably matched unrelated donor cannot be readily identified. A major limitation to the more widespread use of UCB transplantation (UCBT) has been delayed or failed engraftment, which used to be a common problem, particularly in adult recipients. The two most important factors determining the likelihood of neutrophil and platelet engraftment after UCBT are nucleated cell dose and HLA disparity. More recently, it has been shown that transplantation of two UCB units, harvested from separate donors, increases the speed of engraftment and is associated with a decreased risk of primary graft failure. Consequently, results approaching those achievable using a well-matched adult unrelated donor are now routinely observed in patients with high-risk leukaemia after a double UCB transplantation performed using UCB units that meet well-defined cell dose and matching criteria. Immune reconstitution is markedly delayed after UCBT and viral infections (particularly CMV, HHV 6 and adenovirus) contribute to the significant infectious-related TRM although the advent of improved supportive care strategies, notably the use of letermovir (a CMV DNA terminase complex inhibitor) may reduce this risk. The development of novel conditioning regimens, which deliver high rates of durable engraftment, coupled to the modest toxicity UCBT, means that this modality is often the preferred approach in both children and adults,

particularly when performed in transplant centres with sustained expertise in UCBT delivery (Barker *et al.* 2020). Continued progress in the development of *ex vivo* stem cell expansion protocols promises to further increase the number of patients who will benefit from cord blood transplants.

Conditioning regimens: basic principles

The combination of drugs and radiotherapy administered prior to stem cell infusion is termed the 'conditioning' or 'preparative' regimen. In autologous SCT, where there is no alloreactive response, the sole purpose of the conditioning regimen is disease eradication. The most common conditioning regimens used in autologous transplantation use alkylating agents, alone or in combination, and other chemotherapeutic drugs.

In the setting of allogeneic SCT, the conditioning regimen serves two purposes: immunosuppression designed to abrogate an HVG reaction, thereby preventing graft rejection, and dose escalation of chemoradiotherapy in order to de-bulk and potentially eradicate host malignant disease. Total body irradiation (TBI) and high-dose cyclophosphamide have been the most commonly used immunosuppressive modalities in allogeneic transplantation but these are increasingly being either supplanted or complemented by fludarabine and other purine analogues such as clofarabine as outlined below. Of note, the advent of fludarabine-based regimes coupled with the increased use of PBSC as a stem cell source has played an important part in the recent reduction in rates of primary graft failure, particularly in recipients of alternative donor transplants. Historically, conditioning regimens resulted in irreversible myeloablation. Increasing recognition of the potency of the GVL effect, coupled with awareness that the toxicity and attendant TRM of MAC regimens precluded their routine deployment in patients older than 50–55 years led to the development of less intensive conditioning regimens, tolerable in older patients and which rely on a GVL effect as the dominant anti-tumour activity. Associated with a substantially reduced TRM, both non-myeloablative (NMA) (which result in only transient cytopenias) and RIC regimens (which result in significant cytopenias but use dose-reduced chemotherapy schedules) permit allogeneic transplants to be safely performed in patients who would previously been judged ineligible on the grounds of age or comorbidity (Gagelmann and Kroger 2021). The remarkable success of RIC and NMA regimens in reducing early transplant toxicity has resulted in a dramatic increase in the number of allografts performed worldwide and has transformed treatment options for many patients with AML, MDS, ALL and relapsed lymphoma in whom conventional chemotherapy would be unlikely to deliver long-term disease-free survival. Although RIC regimens are associated with a higher rate of disease relapse than would be observed using a MAC regimen, this deficiency is at least partially offset by a marked reduction in early and late TRM. The results of prospective randomised studies comparing outcomes after specific MAC and RIC regimens will be essential in order to assist personalisation of conditioning regimen selection in specific disease settings but are already proving of value in diseases such as AML as outlined below.

Conditioning regimens in autologous SCT

Conditioning regimens in autologous SCT are designed with dose intensification in mind and are limited principally by extramedullary toxicity. High-dose melphalan (200 mg/m^2) is the standard conditioning regimen in myeloma autografts. A randomised comparison between a TBI-containing regimen and high-dose melphalan alone demonstrated that no benefit was associated with the use of TBI. BEAM (carmustine, etoposide, cytarabine, melphalan) is widely used in patients with lymphoma although the BCNU is frequently substituted for lomustine (LEAM) or, in some centres, bendamustine (benda-EAM) albeit the latter demonstrating an increased risk of nephrotoxicity. In patients with relapsed CNS lymphoma, thiotepa-based preparative regimens play an important role. Autologous transplants are indicated in certain patients with AML, principally those with APML in second CR, but sometimes also in patients with AML associated with a CBF abnormality who lack an allogeneic donor, and both busulfan/cyclophosphamide and cyclophosphamide/TBI are effective preparative regimens in this setting. A number of other drug combinations incorporating melphalan, busulfan and thiotepa are used in solid tumours. The major extramedullary toxicities of these regimens are mucositis and gastrointestinal toxicity. Disappointingly, there are few prospective randomised data on which to base the choice of conditioning regimen in autologous SCT.

Myeloablative conditioning regimens in allogeneic SCT

Traditionally, the most widely utilised MAC regimens used in allogeneic SCT employed combinations of cyclophosphamide (Cy) and either TBI or busulfan (Bu). These are now increasingly being supplanted by fludarabine (Flu) based combinations, typically Flu/BU or Flu/Cy.

TBI/cyclophosphamide

The use of high-dose Cy and myeloablative doses of TBI (collectively referred to as Cy/TBI) remains a commonly used MAC regimens in patients allografted for acute lymphoblastic leukaemia (ALL) but is now less commonly employed in patients with AML. Cy is an alkylating agent that, when administered in the doses routinely used in myeloablative conditioning regimens (typically 120 mg/kg), has both immunosuppressive and anti-leukaemic properties. It is a pro-drug that must be metabolised by the cytochrome P450 system in the liver to produce metabolically active derivatives (principally

phosphoramide mustard) which exert their cytotoxic activity through the production of inter-strand DNA links. The two major complications of Cy at the doses employed in allogeneic transplantation are haemorrhagic cystitis and cardiac toxicity. Haemorrhagic cystitis results from the toxic effects of a Cy metabolite, acrolein, on the uroepithelium and can be reduced by use of sodium 2-mercaptoethanesulfonate (MESNA), while cardiac toxicity is rare at doses of cyclophosphamide below 150 mg/kg.

TBI has dual immunosuppressive and anti-leukaemic properties when administered in myeloablative doses (typically 12–14.4 Gy). Radiobiological principles predict that the toxicity of TBI can be reduced by either decreasing the overall dose of radiation or, as is now common, by giving it in fractionated form over a number of days (e.g. 14.4 Gy divided into eight fractions over 4 days). The degree of immunosuppression produced by TBI-containing regimens is related to the total dose of irradiation delivered. The use of higher TBI doses is therefore an effective method of optimising engraftment in myeloablative allografts where there is use of TCD or an alternative donor and consequently an increased risk of graft failure. Haematological malignancies are highly radiosensitive and the risk of disease relapse is reduced if a higher dose of TBI is used, although this benefit is blunted by increased transplant toxicity (see below). Early complications associated with the use of TBI include nausea, vomiting, diarrhoea and parotitis, which can usually be managed symptomatically. Increased doses of TBI are also associated with pneumonitis and veno-occlusive disease (VOD) of the liver, both of which may be life-threatening. Long-term complications include cataract formation, hypothyroidism, infertility and, in children, growth retardation. In patients undergoing a sibling allograft for ALL, etoposide is often substituted for cyclophosphamide and a TBI/etoposide regimen has been reported to be associated with improved outcome.

Busulfan/cyclophosphamide

Busulfan/cyclophosphamide (Bu/Cy) is a well-established MAC regimen which has the practical advantage of not requiring the presence of irradiation facilities on site. Bu is an alkylating agent with potent activity against leukaemic progenitors and is a core component of both allogeneic and some autologous transplant regimens. Historically, Bu has only been available as an oral preparation, used at a dose of 14–16 mg/kg delivered 6-hourly over a period of 4 days. In this formulation, VOD and pulmonary and central nervous system (CNS) toxicity represent major complications. The pharmacokinetics of oral Bu are highly variable because it undergoes first-pass metabolism in the liver and there is therefore substantial interpatient variability in plasma drug levels using a standard dosing schedule. Because the incidence of VOD is closely correlated with higher plasma Bu levels and patients with low Bu levels also have an increased risk of relapse, oral preparations of this drug are far from ideal. The successful development of an intravenous formulation of Bu, characterised by more predictable pharmacokinetics, has substantially improved the tolerability and efficacy of Bu-based MAC (the typical dose of intravenous Bu in MAC regimens is 12.8 mg/kg) and RIC (the typical dose of intravenous Bu in RIC regimens ranges from 6.4 to 9.6 mg/kg) regimens and has largely replaced oral Bu use. It should be remembered that both oral and intravenous Bu preparations require the use of prophylactic anticonvulsants to prevent seizures when used at high dose.

Fludarabine/busulfan and other novel MAC regimens

Flu augments alkylator-induced cell killing *in vitro* and regimens combining Flu are active and well tolerated although often associated with a significant degree of mucositis which often requires opiates for management. Flu in combination with myeloablative doses of Bu (FB4) is now established as an important new MAC regimen. In AML, a randomised comparison of FB4 and BuCy in patients allografted using a matched sibling or unrelated donor demonstrated superiority of outcomes in the FB4 arm with respect to 1-year TRM (reduced from 17 to 8% in the Flu/Bu4 arm) and equivalent overall survival (Rambaldi *et al*. 2015). This has resulted in its widespread adoption by many transplant centres as a preferred MAC regimen in younger fit patients allografted for AML.

Thiotepa is a new alkylating agent which is commonly combined with Bu and Flu in the TBuF regimen as a MAC regimen for patients undergoing a haploidentical transplant. The utility of a TBuF regimen in recipients of allografts from sibling or adult unrelated donors is currently being evaluated in prospective randomised trials. Treosulfan is an additional alkylating agent that shows promise as a component of both MAC and RIC regimens in patients allografted for myeloid disorders and shows particular promise in combination with BU as a novel RIC regimen (Beelen *et al*. 2020).

The increased HLA disparity and high rates of primary graft failure in UCBT make it critical to optimise the immunosuppressive properties of the preparative regimen. In adults undergoing transplantation using a myeloablative regimen, engraftment rates in the region of 90–95% can be achieved by the addition of Flu to a Cy/TBI regimen, providing an adequate stem cell inoculum is used. More recently, highly promising results have been achieved using an 'intermediate intensity' conditioning regimen consisting of Flu, Cy, Thiotepa and 400 cGy TBI (Barker *et al*. 2020).

GVHD prophylaxis strategies in recipients of a MAC allograft

Post-transplant immunosuppression using a calcineurin inhibitor (ciclosporin or tacrolimus) and methotrexate remains a widely used GVHD prophylaxis regimen in patients undergoing

a MAC sibling or unrelated donor transplant. Rates of chronic extensive GVHD using such an approach are in the region of 30 and 66% for recipients of T-replete sibling and unrelated donor transplants, respectively. TCD is an additional, and highly effective method of reducing the risk of both acute and chronic GVHD in patients transplanted using a calcineurin inhibitor-based prophylaxis regimen. TCD in this setting can be achieved either by manipulating the stem cell inoculum *ex vivo* (e.g. by manipulating the graft using immunomagnetic beads to enrich for CD34+ cells or remove T cells) or by the *in vivo* administration of T-cell-depleting antibodies such as anti-thymocyte globulin (ATG) or alemtuzumab (a humanised monoclonal antibody that recognises CD52). Although a highly effective method of GVHD prophylaxis, TCD is associated with an increased risk of relapse and graft failure and delayed immune reconstitution, increasing the risk of post-transplant infections such as CMV and EBV. A number of recent randomised studies, although not all, have confirmed the ability of *in vivo* ATG to reduce the risk of both acute and chronic GVHD without increasing relapse risk in recipients of a matched unrelated donor transplant resulting in its widespread adoption in many European centres. In contrast, there are no compelling randomised data supporting the use of *ex vivo* TCD although a number of centres still use this approach in combination with a markedly intensified TBI-based MAC regimen. PTCy, initially developed in the setting of haploidentical transplantation, has also recently shown promise in patients transplanted using an HLA-matched sibling or unrelated donor. A number of other agents, including abatacept and JAK inhibitors, are under investigation as novel GVHD prophylaxis regimens.

In patients transplanted using a ciclosporin-based GVHD prophylaxis regimen, there is evidence that the intensity of post-transplant immunosuppression is an important predictor of relapse and post-transplant outcome. Great attention should therefore be paid to meticulous monitoring of ciclosporin (and by extension tacrolimus) levels in the post-transplant period and both the degree of post-transplant immunosuppression and its duration patient should be personalised according to an individual patient's predicted risk of relapse. Thus, it may be desirable to avoid the use of TCD in patients with advanced leukaemia in whom the risk of relapse is high. In contrast, patients with a low risk of disease recurrence, such as aplastic anaemia, may benefit from a more intensive GVHD prophylaxis regimen.

Reduced-intensity conditioning regimens in malignant and non-malignant disease

Reduced-intensity regimens which incorporate either Flu or low-dose (200 cGy) TBI as a core immunosuppressive component reliably deliver durable donor stem cell engraftment and are associated with a markedly reduced toxicity profile, particularly in older patients or those with significant comorbidities. The recent demonstration that the risk of relapse after a RIC allograft is both reduced in the presence of chronic GVHD and correlates with the intensity of post-transplant immunosuppression confirms that these transplant platforms also have the capacity to exert a potent GVL effect. However, disease relapse and GVHD remain major causes of morbidity and mortality after RIC allografts and despite a proliferation of differing regimens, there are few randomised data to inform the choice of the optimal RIC regimen.

Low-dose (200 cGy) TBI-based regimens

Pioneering work in a canine model by Storb's group demonstrated that durable engraftment could be achieved with an NMA conditioning regimen that used immunosuppressive doses of TBI (200–450 cGy) in conjunction with post-transplant immunosuppression in the form of ciclosporin and mycophenolate mofetil. Combined administration of Flu and low-dose TBI (200 cGy) reliably achieves high rates of engraftment and full donor chimerism with a substantially reduced TRM, compared with myeloablative preparative regimens in patients with a range of haematologic malignancies up to (and on occasions beyond) the age of 70. The main causes of treatment failure using this regimen include a substantial risk of chronic GVHD and disease relapse, particularly in patients with advanced haematologic malignancies. Up to 66% of patients experience chronic extensive GVHD, the management of which is often very challenging, especially in elderly and more frail patients.

Fludarabine-based RIC regimens

The most commonly used RIC regimens all incorporate an immuosuppressive Flu backbone administered in combination with a range of myelosuppressive drugs including Bu, melphalan, treosulfan and Cy. In common with NMA regimens, RIC regimens deliver long-term disease-free survival in patients with both myeloid and lymphoid malignancies and are associated with markedly reduced immediate transplant toxicity. Commonly used RIC regimens in AML/MDS include Flu in combination with Bu (FB2), melphalan (Flu/Mel) and treosulfan (Flu/Treo). In patients allografted for AML, an early randomised comparison of two of the most widely used NMA/RIC regimens demonstrated equivalent survival in patients transplanted in CR1 using either a FB2 or low-dose TBI regimen (Blaise *et al.* 2013). Of interest, the low-dose TBI regimen was associated with a markedly reduced 100-day transplant-related mortality but this benefit was blunted by an increased risk of disease relapse. Encouraging results have been reported in a randomised trial utilising a Flu/Treo RIC regimen which merit confirmation (Beelen *et al.* 2020). More recently, a UK study demonstrated no benefit of a sequential Flu-amsacrine-cytarabine-Bu regimen compared with FB2

(Craddock *et al*. 2021). On the basis of these data, it is not possible currently to recommend an optimal RIC regimen in older patients with AML. The accelerated delivery of prospective randomised trials, with embedded genomic and MRD assessments which compare specific RIC regimens through trial networks such as the US BMT CTN and UK IMPACT initiatives is important if patient outcomes in this and other transplant settings are to be optimised. Initial RIC regimens utilised a T-replete stem cell inoculum and were associated with a significant risk of severe acute and chronic GVHD. As outlined below *in vivo* TCD using either ATG or alemtuzumab is now a widely incorporated as a highly effective form of GVHD prophylaxis in patients transplanted using a Flu-based RIC regimen.

Conditioning regimens in non-malignant diseases

In non-malignant disorders, such as aplastic anaemia, Cy alone can be used as a conditioning regimen and is sufficiently immunosuppressive to permit engraftment of allogeneic stem cells, provided an adequate stem cell inoculum is transplanted. Addition of Flu is increasingly used in conjunction with Bu coupled with alemtuzumab or ATG in patients with sickle cell disease and thalassaemia.

Strategies for GVHD prophylaxis in RIC regimens

Until recently, two contrasting approaches were used for GVHD prophylaxis in patients undergoing a RIC allograft. The first uses combinations of a calcineurin inhibitor (ciclosporin or tacrolimus) and methotrexate or mycophenolate mofetil (MMF) as the sole form of GVHD in patients transplanted using a T-replete stem cell inoculum. Methotrexate is commonly omitted and replaced by either MMF or sirolimus (an MTOR inhibitor). The alternative approach uses ciclosporin alone in combination with either ATG or alemtuzumab as *in vivo* TCD. Supporters of such a TCD approach argue that the incidence of acute, and particularly chronic, GVHD is otherwise unacceptably high, particularly in older patients transplanted using an unrelated donor. It can also be argued that this strategy generates an effective platform for the subsequent delivery of DLI with less GVHD-related toxicity. They point to the effectiveness of ATG and alemtuzumab in reducing the risk of GVHD while at the same time securing high rates of durable donor engraftment. Those favouring a T-replete strategy highlight the risks of TCD citing a potential abrogation of a GVL effect, which it is argued may increase the risk of relapse, as well as delayed immune reconstitution and an attendant increased risk of viral, particularly CMV, infection. Recently, the BMT CTN 1703 trial has shown that PTCy improves clinical outcomes when compared to a standard T-replete strategy after RIC HLA-matched transplants. PTCy is also now being compared to TCD in prospective randomised controlled trials. The optimal form of GVHD prophylaxis used in an individual patient will depend on a number of factors including stem cell source, the type of RIC regimen used, the underlying disease biology and presence of co-morbidiies.

Clinical management of patients undergoing stem cell transplantation

The use of PBSCs coupled with improved supportive care has decreased the 100-day TRM of autologous transplantation to 1–3% in most centres. The morbidity and mortality of allogeneic SCT have also continued to fall over the last two decades, but although the 100-day TRM after either a RIC or MAC allograft can be as low as 3–6%, the 1-year TRM remains substantially higher and in the region of 15–25%, depending on patient age and comorbidities, donor type, disease status and stem cell source. This progress reflects advances in supportive care and, in patients undergoing an unrelated donor transplant, more accurate tissue typing. Despite these improvements, organ toxicity, acute GVHD and infections consequent on delayed immune reconstitution still represent major causes of morbidity and mortality in the first few months after allogeneic transplantation (Figure 37.4). In addition, chronic GVHD remains an increasing issue for patients and transplant services alike, with the increasing use of mismatched unrelated and cord blood donors. The long-term complications of allogeneic transplantation, which can significantly compromise a patient's quality of life, are also increasingly recognised and have led to the introduction of specific late-effects clinics where multidisciplinary input from transplant physicians, specialist nurses, endocrinologists, gynaecologists, ophthalmologists and psychologists can be provided (Hashmi *et al*. 2018).

Practicalities of stem cell infusion and blood product support

Stem cell products are infused in the same way as other blood products except for the fact that online blood filters should not be used. Stem cell products must not be irradiated. They may either be infused once collected or, in the case of cryopreserved products, be infused immediately after being unfrozen, at the patient's bedside. The most common side-effect of the cryopreservative (dimethyl sulfoxide, DMSO) is nausea, but since it is excreted by the lungs, a garlic-like odour is also observed for 2–3 days after stem cell infusion. Damage to red blood cells releases free haemoglobin, which can precipitate acute renal failure, and thus patients should be adequately hydrated prior to stem cell infusion and for the following hours. In all patients, urine output must be monitored closely.

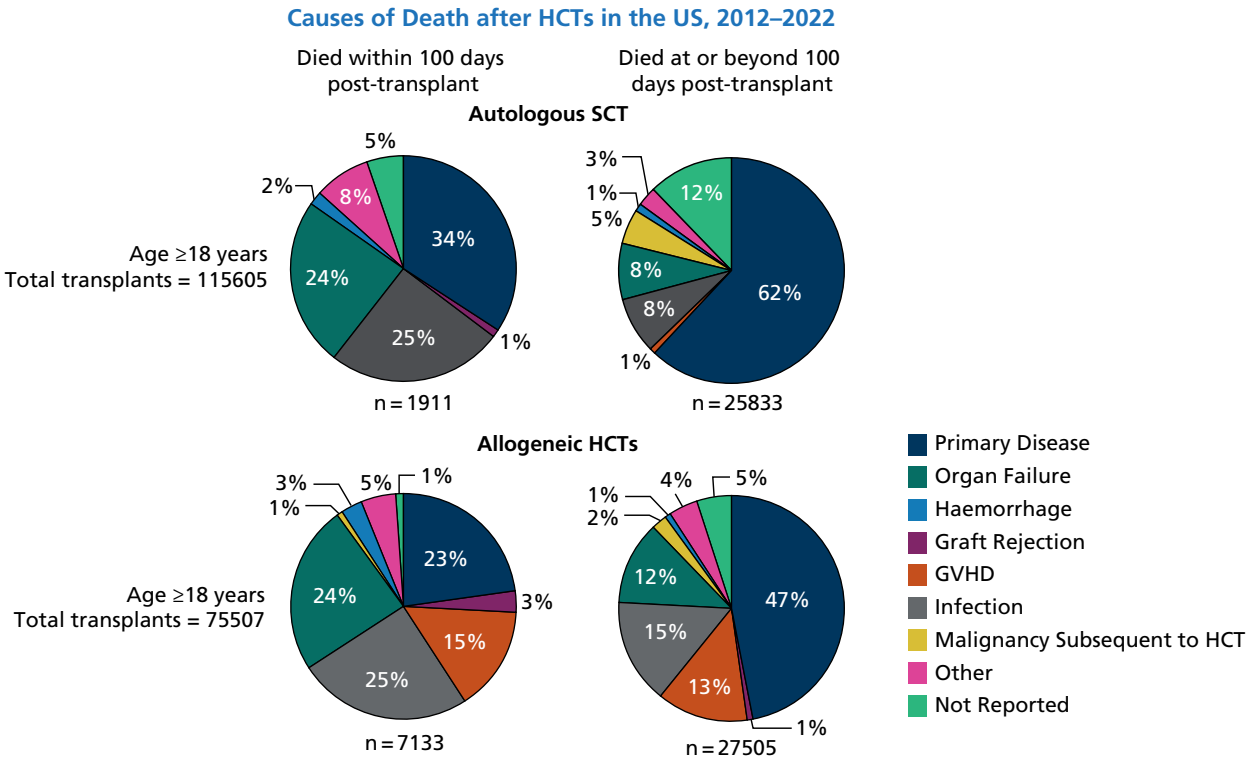


Figure 37.4 Causes of death after stem cell transplantation in (a) autologous and unrelated donor transplants in 2018 (Source: Courtesy of the International Bone Marrow Transplant Registry.)

Patients will require red cell and platelet support during the immediate post-transplant period. In allograft recipients, particularly UCBT and patients receiving myelotoxic drugs such as ganciclovir, platelet transfusion may be required for a number of months after transplantation. All cellular blood products should be irradiated (25 Gy) prior to administration in order to prevent transfusion-related GVHD (see Chapter 19). This should be commenced 6 weeks prior to transplant (and 7 days prior to stem cell collection in the case of autologous transplantation) and is continued for 3 months after an autologous transplant (or 6 months if TBI was used) or indefinitely for allogeneic transplants. In patients undergoing allogeneic SCT where there is a major ABO incompatibility between donor and recipient (e.g. a group O recipient receiving group A bone marrow), the a bone marrow graft must be depleted of red cells prior to administration. When PBSCs are being transplanted, red blood cells are usually effectively depleted during leukapheresis, although on occasions the stem cell product may need to be administered over two days. Measurement of anti-A or anti-B titres, as appropriate, is mandated in all patients undergoing an allogeneic transplant associated with a major ABO incompatibility. The subsequent choice of blood group for platelets or red cells depends on the precise nature of the ABO incompatibility, the time from transplant and the results of blood grouping. For example, if the donor is group A and the recipient is group B, red cells with blood group O would be used until the patient began to type as blood group A, whereas group AB platelets (lacking anti-A and anti-B) would be infused. Delayed erythroid engraftment or haemolysis caused by continuing synthesis of isohaemagglutinins by host lymphocytes may occur several weeks after stem cell infusion and is associated with the presence of a positive direct antiglobulin test and anti-donor red cell antibodies in serum or red cell eluates.

Early complications of allogeneic stem cell transplantation (days 0–90)

Graft failure

Although there are no agreed definitions, primary graft failure will be the most likely cause in the event a patient fails to achieve a neutrophil recovery within 4 weeks of transplant. Risk factors include transplantation of a low CD34+ cell or bone marrow mononuclear cell dose, increased donor–host HLA disparity, the presence of anti-donor HLA antibodies and the deployment of TCD, particularly using *ex vivo* depletion technologies. The mortality of primary graft failure is in excess of 50% and it should be suspected in any who fail to demonstrate evidence of neutrophil engraftment by day +21. Use of PTCy may delay engraftment compared to other protocols and this needs to be considered when deciding on intervention.

The key investigations include an urgent bone marrow aspirate and trephine and chimerism studies. Graft rejection is likely in patients with no morphological evidence of engraftment or leukaemia (as demonstrated by a good-quality trephine biopsy) and an absence of donor chimerism; these patients require a second transplant from an alternative donor, preferably using G-CSF-mobilised PBSC, after administration of a second conditioning regimen incorporating fludarabine with or without low-dose TBI designed to maximise host immunosuppression. When donor cells are identified by chimerism studies, this will imply poor graft function without rejection. Under these circumstances, rescue of graft function can be achieved by a CD34-selected stem cell infusion without any additional conditioning.

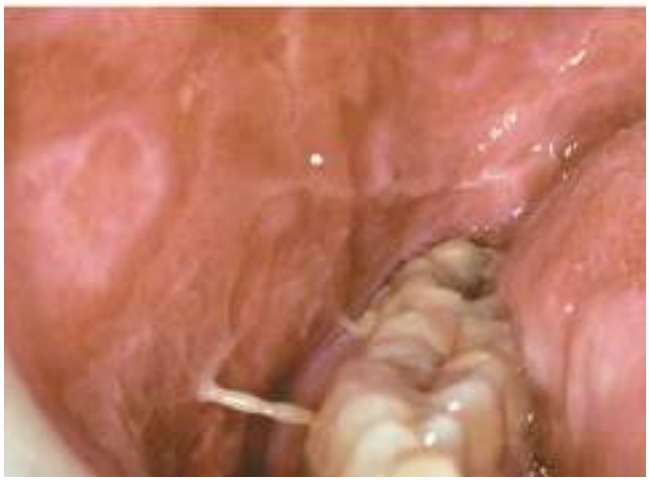
Secondary graft failure leads to one or more cytopenias after initial donor engraftment in the absence of disease relapse. It is rare in sibling allografts and occurs predominantly in recipients of mismatched (including haplo-identical) or unrelated donor transplants. The aetiology of secondary graft failure is often complex, but causes that need to be considered include late graft rejection (rare), major ABO incompatibility, drugs (particularly ganciclovir and, on rare occasions, cotrimoxazole), viral infection (CMV, HHV-6 and parvovirus B19) and, rarely, hypersplenism. Secondary graft failure is associated with significant mortality related to infection and must be investigated urgently with a bone marrow aspirate and trephine, urgent chimerism studies and virological assessment to exclude parvovirus B19 or human herpesvirus (HHV)-6 infection. In patients with a hypocellular marrow and evidence of donor cells on chimerism, a second infusion of CD34-positive selected PBSC can be used and leads to responses in about 2 in 3 patients. Additional measures could include use of growth factors or thrombopoietin mimetics. For single red cell or platelet cytopenias, the possibility of immune haemolytic anaemia or immune thrombocytopenia should also be considered.

Acute graft-versus-host disease

Acute pattern GVHD usually occurs within the first 3 months following transplant, often at or near the time of engraftment, and is characterised by the presence of rash, diarrhoea or (less commonly) abnormal liver function tests. Depending on the conditioning regimen and GVHD prophylaxis employed, between 20 and 70% of patients will develop this disorder. Acute pattern GVHD occurring after 3 months, with no features of chronic GVHD, is also increasingly recognised in patients receiving RIC transplants, often following withdrawal of immunosuppression. Risk factors for the development of acute GVHD include increased recipient age, unrelated or HLA-mismatched donor transplantation and the use of a female donor. Children have a lower risk of acute GVHD and recipients of UCBT may have a lower incidence of GVHD compared with adult bone marrow transplantation with similar degrees of HLA disparity.

Skin GVHD typically presents as a maculopapular rash involving the face, neck, palms and soles, but may extend to involve the whole body (Figure 37.5). In the worst cases, it progresses to erythroderma, with bullae formation and painful blistering. Histology shows apoptosis at the base of dermal crypts, dyskeratosis and evidence of lymphocytes in a perivascular distribution or adjacent to dyskeratotic keratinocytes. Gastrointestinal involvement presents

(a)



(b)

Figure 37.5 Acute skin GVHD: (a) acute cutaneous GVHD; (b) chronic oral GVHD.

with nausea, vomiting, secretory diarrhoea and/or abdominal pain. In more advanced forms, severe abdominal pain and distension associated with voluminous, occasionally bloody, diarrhoea may occur. Gastric, antral and rectal biopsies have a high diagnostic yield, with diagnostic features including the presence of apoptotic cells in the base of crypts and a lymphocytic infiltrate. A well-defined manifestation of upper gut GVHD is the development of anorexia and nausea, both of which usually resolve rapidly if treated with lower dose methylprednisolone (0.5–1 mg/kg). A rising bilirubin and raised alkaline phosphatase are the initial features of liver GVHD, which typically develops later than skin or gut GVHD. Liver histology is diagnostic and demonstrates a portal tract lymphocytic infiltration, peri-cholangitis and bile duct loss.

Accurate and early diagnosis of acute GVHD is essential for effective management of this potentially life-threatening disorder. Where possible, diagnostic biopsies should be taken to both confirm the presence of intestinal or liver GVHD and assist in the exclusion of other aetiologies. It is also important to stage GVHD accurately and the revised criteria devised by the Mount Sinai Acute GVHD International Consortium are widely recommended (Table 37.1). This staging system is a reliable indicator of prognosis and guides the intensity of treatment required. To aid assessment and standardisation, acute GvHD scores can be calculated using an eGvHD App (www.uzleuven.be/egvhd). It is possible that peripheral blood biomarkers of tissue injury (e.g. ST2 or Reg3a) will be used in the future to determine prognosis and guide treatment. Grade 2–4 acute GVHD should be treated with high-dose methylprednisolone (typically 2 mg/kg daily), which is tapered according to response. In the setting of limited skin GVHD and upper gut GVHD, topical or oral steroids (e.g. prednisolone 0.5–1 mg/kg) coupled with optimisation of ciclosporin levels may be sufficient to control symptoms. Approximately 70% of patients will improve significantly with oral or intravenous corticosteroid therapy, but a number will either fail to respond or relapse when immunosuppression is tapered. Failure to respond to 5–7 days of intravenous corticosteroid therapy or failure to taper dosing without disease progression defines steroid-resistant and steroid-dependent acute GVHD, and these patients require the use of second-line therapy. The current standard of care for second-line therapy is the JAK1/2 inhibitor, Ruxolitinib, which is superior to other approaches. Dosing is lower than used for myelofibrosis (10 mg bd), but lower doses may be required in patients with co-existent cytopenias or end-organ dysfunction. Even with the use of ruxolitinib, steroid-refractory acute GVHD has a poor prognosis, with a high non-relapse mortality, which is consequent predominantly on infectious complications. Currently, robust evidence supporting efficacy of third-line

Table 37.1 Acute GVHD scoring – MAGIC criteria (a) clinical staging; (b) clinical grading.

Stage	Skin (active erythema only)	Liver (bilirubin)	Upper GI	Lower GI (stool output/day)
0	No active (erythematous) GVHD rash	<34 micromol/L (or <2 mg/dL)	No or intermittent nausea or vomiting or anorexia	<500 mL/day or <3 episodes/day
1	Maculopapular rash <25% of body surface area (BSA)	34–50 micromol/L (or 2–3 mg/dL)	Persistent nausea or vomiting or anorexia	500–999 mL/day or 3–4 episodes/day
2	Maculopapular rash 25–50% of BSA	51–102 micromol/L (or 3.1–6 mg/dL)		1000–1500 mL/day or 5–7 episodes/day
3	Maculopapular rash >50% of BSA	103–255 micromol/L (or 6.1–15 mg/dL)		>1500 mL/day or >7 episodes/day
4	Generalised erythroderma (>50% BSA) plus bullous formation and desquamation >5% BSA	>255 micromol/L (or >15 mg/dL)		Severe abdominal pain with or without ileus, or grossly bloody stool (regardless of stool volume).

Overall clinical grade (based upon most severe target organ involvement)
Grade 0: No stage 1–4 of any organ
Grade I: Stage 1–2 skin without liver, upper GI or lower GI involvement
Grade II: Stage 3 rash and/or stage 1 liver and/or stage 1 upper GI and/or stage 1 lower GI
Grade III: Stage 2–3 liver and/or stage 2–3 lower GI, with stage 0–3 skin and/or stage 0–1 upper GI
Grade IV: Stage 4 skin, liver or lower GI involvement, with stage 0–1 upper GI

treatments is lacking and wherever possible, such patients should be offered clinical trials. Patients with acute severe GVHD often require intensive supportive measures, including replacement of gastrointestinal losses, parenteral nutrition, pain control and infectious prophylaxis. Maintenance of enteral nutrition should be maintained wherever possible to prevent gut dysbiosis that has the potential to worsen intestinal GVHD.

Infectious complications

Bacterial, fungal, protozoal and viral infections are a major cause of morbidity and mortality after allogeneic transplantation (see also Chapter 55). Host factors include neutropenia, post-transplant immunosuppression and acute or chronic GVHD requiring steroid therapy. Numerous additional factors contribute to delayed immune reconstitution after an allograft and include thymic atrophy in adults, the use of TCD and a suboptimal stem cell inoculum. Infectious complications are a particular challenge after UCBT. The temporal pattern of infectious complications after allogeneic SCT is shown in Figure 37.6. In contrast, autologous transplantation is associated with significantly fewer infective episodes which are predominantly confined to neutropenia-related bacterial and fungal infections in the immediate post-transplant period.

Considerable progress has been made in the prevention and treatment of infectious complications after allogeneic SCT. All patients should be nursed in single rooms, preferably with laminar airflow or high-efficiency particulate air filtration. Data from prospective randomised trials supports the use of triazole antifungals, such as fluconazole 100–400 mg daily, as an effective means of reducing *Candida* infection. Aciclovir (200–400 mg up to 4 times daily) is usually administered to prevent herpes simplex virus (HSV) re-activation. Quinolone antibiotics (e.g. ciprofloxacin 500 mg twice daily) are used by some units to reduce the risk of severe Gram-negative infections, although the evidence supporting this measure is inconclusive and practice should be guided by advice from local microbiologists concerning the prevalence and sensitivity of drug-resistant organisms. Patients should promptly receive cotrimoxazole (e.g. 480 mg twice daily 3 times per week) prophylaxis at the time of neutrophil engraftment (neutrophils $>1.0\times10^9$/L) to prevent *Pneumocystis jirovecii* infection. Importantly, cotrimoxazole also provides effective prophylaxis against infection with Toxoplasma gondii. If a patient demonstrates a convincing allergy to cotrimoxazole, dapsone or atovaquone may be considered as substitutes for cotrimoxazole. Cotrimoxazole is typically discontinued once patients have completed immunosuppression and reconstituted a lymphocyte count of >1.0 × 109/L. It is also important to remember to restart cotrimoxazole prophylaxis in allograft patients in whom immunosuppressive therapy is restarted.

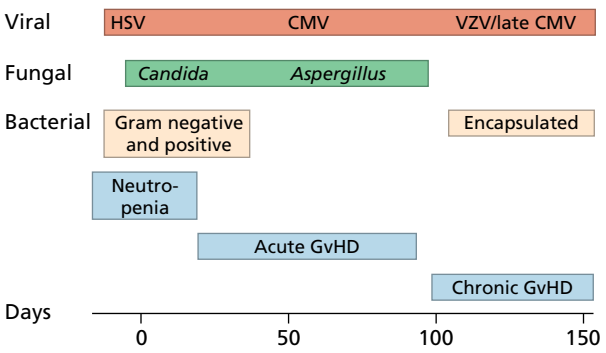


Figure 37.6 Temporal pattern of infectious complications after allogeneic stem cell transplantation.

Allogeneic transplants, particularly recipients of TBI-containing regimens, continue to be at long-term risk from infections caused by encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* and require lifelong prophylaxis with penicillin (250 mg twice daily) or erythromycin (250 mg twice daily) if allergic to penicillin. Antibody titres to diseases for which childhood vaccination is performed decline after SCT. Re-vaccination to bacterial organisms which are targeted by childhood immunisations is therefore recommended, particularly in allograft recipients, and most centres commence such a programme 6–12 months after transplantation.

Herpesvirus infection: CMV, HSV, varicella zoster and HHV-6

Human CMV is a ubiquitous herpesvirus present in up to 60% of the general population, which re-activates after allogeneic SCT in seropositive recipients or those transplanted from a seropositive donor, giving rise to either asymptomatic infection or, less commonly, end-organ damage (CMV disease). Patients at the highest risk of CMV re-activation are seropositive recipients, especially those who have a seronegative donor, or who receive TCD or unrelated donor grafts, and patients who develop GVHD requiring steroid therapy. Previous studies from the EBMT have demonstrated improved transplant outcome in CMV-positive recipients transplanted from a CMV-positive donor, and CMV status remains an important consideration in the choice of an optimal volunteer unrelated donor. In the absence of effective pharmacological prophylaxis, such as can now be provided by letermovir, CMV re-activation occurs in 40–80% of at-risk patients and can result in end-organ damage, typically CMV pneumonitis, but also less commonly upper gastrointestinal ulceration or colitis, hepatitis and retinitis. All at-risk patients should receive weekly monitoring for evidence of CMV infection using a polymerase chain reaction (PCR)-based detection strategy. Until recently, pre-emptive therapy with intravenous ganciclovir, a nucleoside analogue that inhibits viral thymidine kinase, or its oral prodrug valganciclovir was the routine approach to management of asymptomatic CMV infection. While these approaches are effective in reducing the risk of early CMV disease, late CMV infection (beyond 100 days post-transplant)

remains a significant problem, and the use of both ganciclovir and valganciclovir (especially in the immediate post-transplant period) is associated with significant neutropenia and thrombocytopenia. The recent randomised controlled trial demonstrating that prophylactic letermovir, a CMV terminase complex inhibitor, substantially reduces the risk of CMV infection post-transplant and is well tolerated, has transformed prophylactic strategies in patients at risk of CMV infection after an allogeneic stem cell transplant (Marty *et al.* 2017). Letermovir prophylaxis, typically from the time of engraftment to 100 days post-transplant is now routine in at-risk patients, although it is still important to screen such patients for CMV infection until day +100 post-transplant. Late CMV infection after cessation of letermovir may also occur, particularly in patients receiving immunosuppressive therapy for chronic GVHD and prolonged lymphopenia. Primary infection of seronegative patients may occur as a result of the infusion of stem cell or blood products from a CMV-positive donor but is rare. For this reason, seronegative transplant recipients should receive CMV-negative or leucodepleted blood products to limit the possibility of primary infection.

The incidence of CMV pneumonitis after allogeneic transplantation has very substantially reduced since the advent of effective screening and letermovir prophylaxis but remains an important component of the differential diagnosis in patients with post-transplant pneumonitis. It occurs in patients with evidence of CMV reactivation within the first 100 days after transplantation and typically presents with dyspnoea, hypoxaemia and pulmonary infiltrates. Ganciclovir and foscarnet are often ineffective in patients with established CMV pneumonitis. However, recent studies have demonstrated significant activity of cidofovir, which is now considered in some units to be first-line treatment in all patients with CMV pneumonitis. Cidofovir is nephrotoxic but can usually be safely administered if attention is paid to adequate hydration and other nephrotoxic drugs, particularly foscarnet, are discontinued. The role of high-titre CMV immunoglobulin in the treatment of CMV pneumonitis remains unclear, although it is still widely used.

Other members of the herpesvirus family have the potential to cause significant morbidity after allogeneic SCT. The incidence of HSV, which used to be very common in the first 30 days after SCT, has been very substantially reduced by the use of prophylactic aciclovir. Re-activation of varicella zoster virus (VZV) occurs in up to 50% of at-risk patients after allogeneic SCT and typically presents as shingles with severe pain and a dermatomal vesicular eruption. Less commonly, VZV re-activation presents with atypical pain (headache or undiagnosed abdominal pain) in the absence of a rash. The risk of shingles in allograft recipients is substantially reduced by the use of prophylactic acyclovir (e.g. 400 mg b.d) which is typically continued until after patients have received inactivated VZV vaccine (Shingrix) in the post-transplant period. Prompt treatment of shingles or atypical VZV re-activation with high-dose intravenous aciclovir is essential after allogeneic SCT both to reduce the risk of dissemination but also to reduce the severity of post-herpetic neuralgia. HHV-6 is also increasingly being reported in patients receiving TCD allografts or cord blood transplant and typically presents with features of an encephalitis, classically with a psychiatric component including hallucinations, and or hepatitis.

Fungal infections

Fungal infections remain an important complication after both autologous and allogeneic SCT. A high index of clinical suspicion is therefore required in transplant patients, particularly allografts, and most units administer systemic antifungal therapy early in the management of neutropenic fever. Risk factors for the development of fungal infection include prolonged neutropenia after SCT, the use of high-dose corticosteroids for treatment of GVHD and a history of prior fungal infection.

Effective strategies exist for the prophylaxis and treatment of infection with yeasts (*Candida* spp.) and increasingly moulds such as *Aspergillus* spp. *Candida* infections typical manifest as oral thrush and less commonly as oesophageal candidiasis. Hepatosplenic candidiasis is seen occasionally, presenting with high spiking fevers at the time of engraftment in association with abnormal liver function tests. Ultrasound or computed tomography (CT) of the liver and spleen will confirm the diagnosis. Prophylactic use of fluconazole (100–400 mg daily) has proved effective in reducing the incidence of both superficial and invasive candidiasis. Patients who develop either hepatosplenic candidiasis or candidaemia should be treated with systemic antifungals, usually liposomal amphotericin or an echinocandin such as caspofungin. All indwelling catheters must be removed. Emergence of fluconazole-resistant *Candida* species such as *Candida kruseii* or *Candida glabrata* is of concern, and sensitivity data must be requested in any patient with *Candida* infection.

Aspergillus infections usually present prior to or shortly after engraftment. The most common manifestation is as invasive pulmonary aspergillosis (IPA), which typically presents with an antibiotic-resistant fever, a significantly raised C-reactive protein, and abnormal chest radiography or high-resolution CT, often in the absence of respiratory symptoms. Rarely invasive *Aspergillus* infections can present with cerebral or hepatic disease. Accurate diagnosis of *Aspergillus* infections remains problematic since spores are only rarely cultured from lavage fluid or infected tissues and the sensitivity and specificity of other currently available diagnostic techniques is low. Galactomannan detection assays and aspergillus PCR are of value in supporting a diagnosis of *Aspergillus* infection in patients with typical clinical features but all too often results of these sophisticated diagnostic assays are not available in a timely manner and therapeutic decision-making is based on clinical and radiographic features. Operationally, the most

helpful test in deciding whether IPA is a clinical possibility is high-resolution CT of the chest, which should be obtained in all patients with a neutropenic fever that has persisted for more than 72 hours. While the characteristic radiographic features of peripheral nodular shadows, with or without evidence of cavitation or a 'halo' sign, may take weeks to develop, the presence of any significant pulmonary infiltrate substantially increases the likelihood of *Aspergillus* infection and is an indication for the consideration of treatment doses of liposomal amphotericin or a broad-spectrum azole, usually voriconazole. Importantly, the likelihood of IPA in a patient with a normal chest on high-resolution CT is low. Improved antifungal prophylaxis and treatment now make it possible to contemplate allogeneic transplantation in patients with a previous proven or suspected invasive fungal infection. Risk factors for recrudescence of fungal disease in this setting include a short period (<6 weeks) of antifungal treatment prior to transplant, the use of bone marrow or UCB as opposed to PBSCs, occurrence of >grade 2 acute GVHD, and CMV re-activation. Azoles, typically voriconazole, posaconazole and isovuconazole and liposomal Amphotericin B preparations remain mainstays of treatment of probable or proven *Aspergillus* infections post-transplant. While prophylactic strategies in patients undergoing allogeneic transplantation differ, there is good evidence that posaconzole prophylaxis reduces the risk of invasive fungal infections in patients receiving steroid therapy for severe GVHD disease.

Organ toxicity

Gastrointestinal toxicity

Mucositis is frequently observed in patients transplanted using a MAC regimen but is less common in patients transplanted using a RIC regimen. Symptoms of oral pain and pain on swallowing typically develop in the first few days after stem cell infusion and peak approximately 8 days post-transplant. The severity of mucositis is closely correlated with the intensity of the conditioning regimen, the use of higher doses of fractionated TBI and the inclusion of methotrexate as a component of the GVHD prophylaxis regimen. Patients with severe mucositis should receive adequate (often opiate) analgesia and be monitored for evidence of airway obstruction. In patients who develop symptoms of severe oesophagitis, the possibility of superadded infection with *Candida albicans* or HSV must be considered, the latter typically being associated with intractable vomiting.

Diarrhoea and/or abdominal pain are common manifestations of conditioning toxicity. Alternative causes of diarrhoea in the first 4 weeks after transplantation include enteritis due to *Clostridium difficile*, adenovirus, rotavirus, norovirus or CMV infection, acute GVHD and pancreatitis. VZV re-activation can occasionally present with severe abdominal pain, often in association with markedly deranged liver function tests sometimes days in advance of, or even in the absence of, the development of classic vesicles. It is particularly important to exclude a diagnosis of lower gut GVHD in patients who develop diarrhoea within the first 100 days post-transplant. While exclusion of infectious causes, particularly *Clostridium difficile* infection, is extremely important it is sometimes necessary to admit patients so that intravenous methylprednisolone (2 mg/kg) can be started and a sigmoidoscopy and diagnostic biopsy obtained after a short period of observation before the results of stool cultures are available.

Liver toxicity

Abnormalities in liver function tests are commonly observed after allogeneic SCT and causes include VOD, drugs, infectious complications, cholelithiasis, acute GVHD and transfusional iron overload (Table 37.2). VOD is a clinical syndrome characterised by a triad of hyperbilirubinaemia (bilirubin >34 μmol/L), weight gain (>5% over baseline) and painful hepatomegaly. It is associated with evidence of damage to sinusoidal endothelial cells and hepatocytes and subsequent damage to the central veins in zone 3 of the hepatic acinus. In severe cases, hepatic venular occlusion and widespread zonal disruption may lead to portal hypertension, hepatorenal syndrome, multiorgan failure and death. Risk factors for the development of VOD include the use of conditioning regimens containing Bu or higher doses of TBI, pretransplant abnormalities of liver function tests, previous abdominal irradiation and recent exposure to the anti-CD33 and anti-CD22 monoclonal antibodies gemtuzumab ozogamicin (Mylotarg) and inotuzumab ozogamicin respectively. A diagnosis of VOD is most commonly made on clinical criteria. Hepatic Doppler studies demonstrating evidence of reversal of portal flow support the diagnosis. Definitive diagnosis requires trans-jugular venous liver biopsy, which has significant morbidity in the early post-transplant period and is

Table 37.2 Causes of abnormal liver function tests after allogeneic SCT.

Precipitant	Clinical presentation
Veno-occlusive disease	Hyperbilirubinaemia associated with weight gain, ascites and painful hepatomegaly
Drugs	Ciclosporin (hyperbilirubinaemia), azole antifungals (raised AST/ALT)
Haemolysis	Fall in Hb associated with unconjugated hyperbilirubinaemia and positive Coombs test
Biliary obstruction	Dilated biliary tree associated with gallbladder 'sludge' or cholelithiasis
Infection	Viral hepatitis, fungal infection (disseminated aspergillosis or candidiasis), cholangitis lenta (progressive development of obstructive picture occurring days after an episode of sepsis)

therefore often avoided. It is important to exclude other causes of hyperbilirubinaemia, particularly ciclosporin toxicity (which can present with a very similar clinical picture), haemolysis (typically consequent on donor–recipient ABO mismatch or transplantation-associated microangiopathic haemolytic anaemia) and the hepatitis of sepsis (cholangitis lenta). Defibrotide (25 mg/kg/day in 4 divided doses) should be commenced in conjunction with meticulous fluid balance, judicious use of diuretics and, where necessary, haemofiltration, in patients with a likely diagnosis of VOD. Because of the clinical similarities between VOD and ciclosporin toxicity, it is wise to discontinue ciclosporin for at least 48 hours in any patient in whom a diagnosis of VOD is suspected and it is surprising how often hyperbilrubinemia responds to this simple manoeuver.

Renal toxicity

The importance of the daily monitoring of weight, fluid balance and renal function in the effective management of patients after SCT cannot be overestimated. Impairment of renal function is frequently observed after allogeneic SCT unless careful attention is paid to fluid balance and the nephrotoxic potential of drugs commonly used during SCT, especially ciclosporin, amphotericin, aminoglycosides and loop diuretics. Ciclosporin-related renal toxicity is usually easily reversible by temporary omission and dose reduction. Occasionally, ciclosporin toxicity manifests itself as a microangiopathic haemolytic anaemia (MAHA), with features of thrombotic thrombocytopenic purpura/haemolytic uraemic syndrome. Withdrawal of the drug is mandatory. There is no convincing evidence that plasmapheresis is beneficial. Introduction of an alternative immunosuppressive regimen (typically mycophenolate mofetil) in conjunction with oral corticosteroid is required in patients who develop ciclosporin-induced MAHA.

Pulmonary infections and non-infectious complications

A range of pulmonary infections occur after allogeneic SCT. In the first month after transplantation, bacterial and fungal pneumonias are common. CMV, respiratory syncytial virus, influenza and parainfluenza are important causes of pneumonitis and typically occur in the first 90 days after transplantation. *Pneumocystis* pneumonia and *Toxoplasma* infection are still seen occasionally in the first few months after transplantation in patients who do not receive, or who are not compliant with, cotrimoxazole prophylaxis.

Non-infectious pulmonary complications occurring after allogeneic transplantation can be reversible, but demand prompt diagnosis if treatment is to be effective. Pulmonary oedema consequent on either increased capillary hydrostatic pressure caused by fluid overload or increased capillary permeability due to irradiation or sepsis is frequently seen in the post-transplant period. Idiopathic pneumonia syndrome, defined as diffuse lung injury occurring after SCT for which no infectious or non-infectious aetiology can be identified, typically occurs 30–50 days after transplantation. The classic presentation includes dyspnoea, non-productive cough, hypoxaemia and non-lobar infiltrates on chest radiography and can progress rapidly to acute respiratory distress syndrome. Treatment is supportive but frequently unsatisfactory, and steroids have little effect on outcome. Diffuse alveolar haemorrhage is seen predominantly in patients undergoing autologous SCT, but can also be seen in allogeneic recipients. This complication usually occurs within the first 2–3 weeks of transplantation and presents with dyspnoea, non-productive cough and hypoxaemia. Radiographic changes include interstitial or alveolar shadowing. Definitive diagnosis requires bronchoscopy, which shows fresh blood on repeated lavage. Early recognition of this disorder is essential since early intervention with high-dose steroids may significantly improve survival.

Post-transplant lymphoproliferative disease

Post-transplant lymphoproliferative disease (PTLD) includes a spectrum of EBV-driven B-cell hyperproliferative states that range from polyclonal benign proliferations to life-threatening neoplastic disease. Most cases of PTLD involve EBV-seropositive donors and present with lymphadenopathy and fever. In contrast to the PTLDs that develop following solid organ transplantation, the majority of cases after SCT are of donor origin and arise as a result of inadequate T-cell control of proliferation of EBV-infected B cells. Risk factors for the development of PTLD include TCD, particularly the use of ATG and alemtuzumab, and increased HLA disparity. Weekly PCR quantitation of EBV viraemia is indicated in patients undergoing allogeneic transplantation that incorporates ATG or alemtuzumab and in all UCBT procedures until 100 days post-transplant. Patients with a rising EBV load may be managed by a reduction in immunosuppression and/or treated with pre-emptive anti-CD20 monoclonal antibody (rituximab), although the triggers for intervention differ between centres. In patients with a documented PTLD, early treatment with rituximab and, where possible, cessation of immunosuppressive therapy is the key to treatment. DLI using EBV-specific cytotoxic T lymphocytes has also been used with effect. Chemotherapy in patients with rituximab-resistant PTLD is rarely of value.

Intensive care support

The likelihood of developing organ failure requiring admission to the intensive therapy unit (ITU) after SCT varies widely according to stem cell source (autologous versus allogeneic), the intensity of the conditioning regimen and the presence of pre-transplant comorbidities. While recent studies suggest a reduction in the proportion of transplant recipients requiring ITU admission, it remains the case that approximately 10% of allografted patients, and in some institutions significantly more, will require ITU admission at some stage during their treatment.

Analysis of outcomes in patients admitted to ITU has identified factors determining survival at varying stages after transplantation, allowing rational decisions about the likelihood of benefit of sustained intensive care in patients with multiorgan failure. Further sophistication in defining who will benefit from admission to ITU has been provided by the application of scoring systems. These include the Acute Physiology and Chronic Health Evaluation (APACHE) II and III mortality prediction models and Simplified Acute Physiology Score (SAPS) prognostic systems. Application of these scores prior to admission to ITU and, where appropriate, after a 72-hour period on ITU is helpful in identifying patients who will benefit from institution or continuation of intensive support. A number of studies have indicated that the development of progressive organ failure or the presence of multiorgan failure 72 hours after admission to ITU predicts an extremely poor outcome. The careful integration of these data into discussion with relatives and, where possible, patients can spare many the indignity of prolonged and futile intervention.

Late complications of allogeneic stem cell transplantation (beyond day 100)

As the results of allogeneic SCT have improved, its long-term complications have come to be better recognised. Chronic GVHD, secondary malignancies and growth and fertility disorders represent an increasingly important cause of morbidity in patients who have been cured of their underlying disease (Battiwalla *et al.* 2017).

Chronic GVHD

Chronic GVHD refers to a complex syndrome usually occurring more than 3 months following allogeneic transplantation and is its commonest long-term complication. There is increasing recognition that the onset of chronic GVHD onset can be variable and also that patients may present features of both acute and chronic GVHD (often referred to as overlap syndrome). Registry data show that chronic GVHD occurs in approximately one-third of patients undergoing T-replete transplants from *HLA*-identical siblings, rising to two-thirds of patients receiving T-replete grafts from unrelated donors. Chronic GVHD is the major factor that patients report impairs their quality-of-life in the longer term. Risk factors for the development of chronic GVHD include increased recipient age, the use of PBSCs as opposed to bone marrow as the stem cell source, transplantation from an unrelated or *HLA*-mismatched donor, the use of a T-replete stem cell inoculum and the presence of prior acute GVHD. Chronic GVHD may develop directly from acute GVHD (progressive), after the resolution of an episode of acute GVHD (quiescent), or *de novo* in patients with no history of chronic GVHD. The clinical manifestations are characterised by features of both immunodeficiency and concurrent inflammation (Figure 37.7). Patients with progressive-type onset, extensive skin involvement, thrombocytopenia or bronchiolitis obliterans have a particularly poor prognosis. The NIH scoring system based on the number of organs involved and the severity within each affected organ is the current accepted standard. To aid assessment and standardisation, chronic GvHD scores can be calculated using an eGvHD App (www.uzleuven.be/egvhd).

Current treatment involves immunosuppression with ciclosporin or prednisolone prior to the institution of a gradual reduction in responding patients. In patients, who fail to respond or who show steroid dependency, the second-line therapies include Belumosudil (a ROCK inhibitor) or Ruxolitinib. It should be noted that only a small minority of patients receiving these second-line drugs show complete remissions and there continues to be an unmet need for better therapies. Extracorporeal photopheresis can be beneficial in patients with sclerodermatous involvement but appears to be less effective in patients with visceral disease. Wherever possible, refractory patients should be offered clinical trials.

Secondary malignancies

The incidence of solid tumours in recipients of allogeneic SCT is increased compared with control populations and includes a higher rate of skin or buccal cavity squamous cell carcinoma and melanoma. In the largest study to date, the cumulative

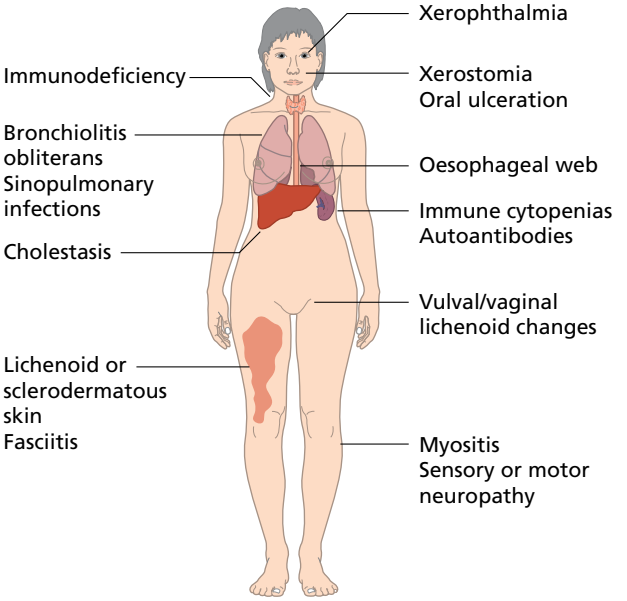


Figure 37.7 Clinical manifestations of chronic GVHD.

incidence of all solid tumours was 2.2% at 10 years and 6.7% at 15 years, although the latter figure may represent an overestimate. Risk factors for the development of secondary malignancies include the use of TBI in the conditioning regimen and the presence of chronic GVHD.

Growth, puberty and fertility

Retarded growth is seen predominantly in children who receive transplants at a younger age (<10 years) and in those who have received irradiation, particularly cranial irradiation. Growth retardation is multifactorial and although growth hormone levels may be diminished as a consequence of hypothalamic or pituitary irradiation, this does not entirely account for the observed reduction in height. Gonadal failure (both testicular and ovarian) is a common consequence of myeloablative conditioning regimens, particularly those that contain TBI or Bu. While prepubertal females receiving Cy alone (as for transplantation in severe aplastic anaemia) have a high probability of experiencing a normal puberty, most receiving other preparative regimens will fail to regain normal ovarian function and will require sex hormone replacement therapy for the induction of puberty. Thereafter sex hormone replacement is indicated to maintain the menstrual cycle and normal bone turnover/mineralisation, but the optimal duration of therapy and the potential long-term risks of hormone replacement therapy in this setting are unknown. Although about 10% of women who receive TBI-containing regimens may have some recovery of gonadal function, the overall incidence of pregnancy is very low. The advent of RIC raises the prospect that some women may remain fertile after transplantation and in this case prior therapies and age will be additional factors determining fertility. Where possible, the need for cryopreservation of sperm in males or oocyte/ovarian tissue cryopreservation in females should be considered at diagnosis before chemotherapy and transplantation..

Complications of autologous SCT

The morbidity and mortality of autologous SCT are much lower than that observed in allogeneic transplantation. The major toxicities relate to immediate or delayed organ failure caused by the conditioning regimen or complications consequent on neutropenia or thrombocytopenia (Table 37.3). The commonest infectious complications are bacterial and fungal and occur in the main in the first 28 days after transplantation. Late complications are rare, but include pulmonary fibrosis in patients who have received Bu and carmustine, and gonadal failure in recipients of TBI or busulfan-containing regimens. Female recipients of BEAM chemotherapy may recover some gonadal function. The development of myelodysplasia is a well-recognised late complication, particularly in patients transplanted using a TBI-based regimen. It appears that prior chemotherapy is an important factor in the development of MDS or AML since the leukaemia-associated cytogenetic abnormality, most commonly involving chromosome 5 or 7, is frequently detected in the infused stem cell inoculum.

Table 37.3 Immediate complications of autologous SCT.

Organ	Complications
Lung	Diffuse alveolar haemorrhage, interstitial pneumonitis especially in recipients of bleomycin/carmustine-and TBI-containing regimens
Gastrointestinal	Mucositis, nausea, diarrhoea
Hepatic	Asymptomatic elevation of bilirubin or AST, veno-occlusive disease (especially recipients of busulfan-containing regimens)
Renal	Prerenal acute renal failure, renal toxicity (nephrotoxic antibiotics), interstitial nephritis
Cardiac	Arrhythmias, cardiac failure (cyclophosphamide)

The evolving role of RIC transplant regimens-which patients should still receive a MAC regimen?

Outcomes in patients after allogeneic stem cell transplantation have improved steadily over recent decades. While considerable progress has been made in reducing transplant-related mortality, largely attributable to the advent of RIC regimens and improvements in supportive care and a reduced risk of severe GVHD consequent upon improvements in tissue typing, there has been little progress in reducing the risk of disease relapse which continues to represent the major cause of transplant failure. The risk of disease relapse is determined by a number of factors including disease biology and status at the time of transplant as well as the intensity of the conditioning regimen. Specifically, in patients allografted for AML, pre-transplant MRD status is an important predictor of relapse risk post-transplant (Hourigan *et al.* 2020; Craddock *et al.* 2021).

It is increasingly recognised that the intensity of the conditioning regimen (MAC versus RIC) is an important determinant of transplant outcome. Large registry studies show that the use of a RIC regimen is associated with a lower TRM but that this benefit is potentially offset by an increased risk of disease relapse. Nonetheless, these retrospective studies raised the important question of whether such RIC regimens should be adopted as the standard conditioning regimen in all patients regardless of age. A number of prospective randomised studies have been addressed this important question in patients with

AML/MDS. To date, randomised data suggest the use of a MAC regimen is particularly important in fit adults with AML in CR1 who are MRD positive and there are no compelling data against using a MAC regimen in fit younger adults regardless of pre-transplant MRD status. At the same time, it is clear that in patients over 50, with some exceptions, the use of a MAC regimen can be associated with excessive toxicity and such patients should be transplanted using a RIC regimen. There is no evidence that delaying transplantation to permit the delivery of further courses of intensive chemotherapy in patients in CR1 who are MRD positive is of benefit. Rather strategies with the potential to reduce the risk of relapse, particularly in patients transplanted using a RIC regimen, are required. Importantly, there is emerging evidence that in patients transplanted for lymphoid diseases such as ALL or Hodgkin lymphoma there may be advantages associated with the use of a RIC regimen in younger patients. Underpinning all decisions concerning the use of either a MAC or RIC regimen is a meticulous assessment of patient fitness. The last decade has seen considerable progress in the development of scoring systems that permit the relatively accurate prediction of TRM after allogeneic transplantation. While the degree of patient–donor HLA mismatch and stem cell source are important determinants of TRM, it is becoming increasingly clear that patient comorbidities are a critical determinant of outcome (Sorror *et al*. 2014). The haemopoietic cell transplant comorbidity index (HCT-CI) incorporates a range of patient-specific variables and predicts outcome after both MAC and RIC allografts. Thus, decisions concerning whether to proceed to an allogeneic transplant are increasingly viewed as a patient-specific appraisal of the predicted reduction in relapse risk if patients are allografted compared with the predicted TRM on the basis of the calculated HCT-CI, stem cell source and donor–patient HLA disparity.

Indications for transplantation

SCT is now an essential constituent of the treatment strategy for many haematological malignancies. In the case of autologous transplantation, current practice has been informed by the results of randomised controlled trials and registry studies comparing the outcome of transplanted patients with those who have received standard treatment. Improvements in the efficacy of first- and second-line treatments in lymphoma coupled with the development of CAR-T cell therapy mean that the role of autologous SCT is evolving rapidly and national guidelines are likely to change. Similarly, the treatment landscape in myeloma has become more complex with the advent of new therapies but autologous transplantation remains, at present at least, the standard of care for patients who respond to first-line therapy. Generation of prospective randomised trial data to inform the optimal role of allogeneic transplantation in both lymphoid and myeloid diseases has proved more challenging for a number of issues. Obstacles not only include randomisation biases introduced because of the perceptions of either physicians or patients, but also the very real problem that the allograft arm may include a smaller proportion of patients with high-risk disease, given the propensity of such patients to relapse before they reach transplantation. The only effective approach that allows these biases to be removed has been the use of a 'donor versus no-donor' analysis, which exploits the availability of an HLA-identical sibling donor as a form of biological randomisation. However, such an approach is hard to apply in rarer diseases and when most patients potentially have access to an alternative stem cell donor. Consequently, despite the development of novel statistical methodologies such as the Mantel–Byer analysis, many of the recommendations concerning the role of allogeneic transplantation in specific diseases will continue to be based on registry data or phase II studies. Prospective randomised trials are urgently needed in allogeneic SCT patients in order to improve transplant outcomes and generation of such robust datasets is necessary in order to inform key therapeutic areas such as conditioning regimen choice, design of GVHD prophylaxis regimens and the generation of innovative strategies with the potential to reduce the risk or relapse post-transplant.

With the aim of providing a robust framework for patient selection for both autologous and allogeneic transplantation, the EBMT publishes a regularly updated summary of indications for transplantation based on currently available data (Table 37.4), which is widely used to inform clinical practice (20). This categorises specific diseases according to whether autologous or allogeneic. SCT is accepted as standard therapy, is a promising treatment modality requiring further evaluation, or is not indicated.

Thanks to the rapid expansion of unrelated donor registries, over 40 million unrelated donors are now registered worldwide. As a result, it is possible to identify a suitable donor in more than 75% of whites of European descent, although this figure is much lower in other ethnic groups. Preferably the donor should be matched for *HLA A, B, C; DRB1* and *DQB1* (10/10). Initial results with unrelated donor transplantation were associated with a high TRM and an increased incidence of severe GVHD. In the last decade there has been a marked improvement in outcome of unrelated donor SCT because of the availability of high-resolution molecular typing at class I and II loci of the HLA complex. As yet, we lack sufficiently robust information to allow us to identify whether specific allelic mismatches are of particular clinical significance. Although the recent growth in the size of unrelated donor registries internationally has increased the availability of volunteer unrelated donors, their unrepresentative ethnic composition limits the availability of donors for ethnic minorities. In addition, lack of availability of a potential donor (e.g. through being lost to contact) remains a significant problem and affects some minority populations

Table 37.4 Indications for SCT.

		Allogeneic SCT	
	Autologous SCT	**Sibling transplant**	**VUD transplant**
AML first CR			
Favourable risk MRD −ve	GNR	GNR	GNR
Favourable risk MRD +ve	GNR	S	S
Standard-risk cytogenetics (predicted risk of relapse >45% if treated with chemotherapy alone	CO	S	NR
Poor-risk cytogenetics	GNR	S	S
AML second CR	CO	S	S
ALL first CR			
Ph−ve, standard risk MRD−ve	GNR	GNR	GNR
Ph−ve, standard risk MRD+ve	GNR	S	S
Ph-ve, CR1, high risk	GNR	S	S
ALL t(9;22)	GNR	S	S
ALL second CR	S	S	S
CML first CP failing 2nd or 3rd line TKIs	GNR	S	S
CML Accelerated Phase, 2nd CP	GNR	S	S
Myelofibrosis: Primary or secondary with intermediate of high DIPSS score	GNR	S	S
MDS Very low and low risk (IPSS-R)	GNR	CO – if significant cytopenias	CO- if significant cytopenias
MDS Intermediate risk (IPSS-R)	GNR	CO	CO
MDS High, Very High Risk	GNR	S	S
CMML	GNR	CO	CO
Myeloma Standard risk	S	CO	GNR
Myeloma High Risk	S	CO	CO
Hodgkin lymphoma first CR	NR	NR	NR
Hodgkin lymphoma relapsed	S	S (if relapse post auto-SCT)	CO (if relapse post-SCT)
NHL DLBCL first CR	D	GNR	GNR
NHL DLBCL relapse-note emerging role of CAR-T cell therapy in this setting	S	CO	CO
NHL follicular	GNR	GNR	GNR
NHL follicular chemo-sensitive relapse	S	CO	CO
Severe aplastic anaemia	NR	R	CO
Haemoglobinopathies	NR	S	D
Multiple sclerosis	D	GNR	GNR

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; CO, clinical option; CR, complete remission; D, developmental; DLBCL, diffuse large B-cell lymphoma; GNR, generally not recommended; MDS, myelodysplastic syndrome; MRD, measurable residual disease; NHL, non-Hodgkin lymphoma; Ph, Philadelphia chromosome;S, standard of care; VUD, volunteer unrelated donor. (*Source:* Snowden *et al.* (2022). Reproduced with permission of Nature Publishing.)

disproportionately. Consequently, the timely identification of a suitable alternative donor remains a challenge for a significant number of patients, particularly those with high-risk acute leukaemia who require urgent transplantation.

UCB has long been known to be a rich source of haemopoietic progenitors and recent results have demonstrated that high rates of durable engraftment can be achieved in adults, comparable to those achieved in children, providing the cell

dose is optimised. Importantly, it appears that greater degrees of HLA mismatch can be tolerated using UCB than with a volunteer unrelated donor. As a result, it is possible to transplant a cord blood unit mismatched at two or fewer HLA-A, -B or -DRB1 antigens, as long as an adequate cell dose is available. This, coupled with the speed of procurement of UCB units, resulted in the emergence of cord blood as an important alternative stem cell source in both adults and children. Although initial results using UCB in adults were compromised by delayed engraftment and a high rate of primary graft failure, these problems have largely been overcome by ensuring the use of a high total nucleated cell dose or two cord blood units. UCB therefore remains a potentially important stem cell source in adults with high-risk leukaemia lacking a suitable unrelated donor, providing strict matching and cell dose requirements are adhered to, although its precise role needs to be defined in prospective clinical trials.

Historically, haploidentical transplants were associated with unacceptable rates of graft failure and GVHD because of the presence of HLA disparity at all major HLA antigens. Recent studies from groups have shown the feasibility of T-cell-replete haploidentical SCT followed by alloreactive T-cell purging *in vivo* using a strategy of PTCy. Accumulating data have demonstrated encouraging outcomes, both in terms of acceptable transplant toxicity and leukaemia-free survival, in both adults and children transplanted using a haploidentical donor in conjunction with post-transplant cyclophosphamide. Randomised data such as that generated by the recent US BMT CTN network are required however before the role of haploidentical transplantation can be confidently established in patients with high-risk haematological malignancies who have alternative stem cell donors (Fuchs *et al*. 2021).

Prevention and management of disease relapse

Relapse after autologous SCT is caused by either resistance of the underlying haematological malignancy to the conditioning regimen or infusion of contaminating tumour cells at the time of transplantation. While there is trial evidence that a second ASCT can be beneficial in some patients in myeloma, it is likely that this option will need to be re-evaluated in the context of the newer therapeutic approaches available for this disease. Similarly, while patients with lymphoma relapsing after ASCT are often considered for salvage with allograft, other options including CAR-T cells or checkpoint inhibitor drugs are likely to change the therapeutic landscape and treatment algorithms significantly.

Disease relapse remains the commonest cause of treatment failure after allogeneic transplantation and is caused by either resistance of tumour cells to the conditioning regimen or failure of the donor immune system to eradicate residual malignant haemopoiesis. Documented mechanisms for tumour cells evading a GVL effect include downregulation of HLA class II expression on acute myeloid leukaemia blasts or upregulation of co-inhibitory ligands, changes to the tumour microenvironment promoting accumulation of regulatory cell populations (e.g. myeloid suppressor cells or regulatory T cells), the location of residual cells in immune-privileged sites such as the CNS or testes, or loss of T cell 'fitness'.

While it is possible to reduce the risk of disease relapse after an MAC allograft by increasing the intensity of the preparative regimen, this approach has not yet translated into improved overall survival because of the increased TRM associated with a more intense conditioning regimen. In patients transplanted using an RIC regimen, there is limited room for increasing the intensity of the conditioning regimen without increasing transplant toxicity, although this is the subject of ongoing clinical trials including the possible option is the use of targeted radioimmunotherapy as part of the conditioning regimen. Currently, there is more evidence that outcomes can be improved by increasing tolerability and reducing TRM (e.g. through use of different drug combinations or newer drugs such as treosulfan) than by reducing the risk of relapse.

Outside the potential use of new conditioning approaches, reducing relapse requires boosting the GVL effect. Possible measures include limiting post-transplant immunosuppression and correction of mixed T-cell chimerism which is linked to higher rates of relapse, for example in lymphoproliferative disorders. On the basis that it is an indicator of bidirectional tolerance and therefore permissive for disease relapse, the use of pre-emptive donor lymphocyte (DLI) infusions may be used, although definitive evidence that this strategy is effective is being tested in randomised clinical trials. Alternative strategies with the potential to reduce the risk of disease relapse include the administration post-transplant of disease-modifying agents such as tyrosine kinase inhibitors in the setting of AML associated with an *FLT3 ITD* mutation (e.g., sorafenib) or Ph+ leukaemias (e.g., dasatinib) or lenalidomide in patients allografted for multiple myeloma. Post-transplant azacitidine is also being investigated in patients allografted for high-risk AML, given its ability *in vitro* to upregulate tumour antigens and potentially augment a GVL response.

The management of patients who relapse after allogeneic transplantation is complex. In many patients who relapse early after transplant, management should often be palliative, although some patients may want to consider entering early-phase clinical trials. For patients who relapse later or who have indolent disease (e.g. patients with low-grade lymphomas), a DLI or a second transplant could be considered. Two factors determine the use of DLI in clinical practice. Firstly, the ability of DLI to deliver durable remissions is highly dependent on the underlying haematological malignancy. Thus, up to 80% of patients who have relapsed after an allograft for CML will achieve a durable molecular remission with DLI, in contrast to patients transplanted for acute

leukaemia or myeloma, where response rates are much lower. Secondly, additional therapy prior to DLI (e.g. chemotherapy, antibody or small molecule) and acquisition of clinical response, may increase the likelihood of achieving a durable response to DLI. The major complications of DLI are GVHD and less commonly, myelosuppression. Myelosuppression is most commonly observed in patients with overt haematological relapse and may require treatment with G-CSF and occasionally transfusion of donor CD34-positive selected donor PBSCs. In early studies, DLI was administered as a single bulk infusion and was associated with a significant risk of severe GVHD. However, the recognition that use of an escalating schedule of administration substantially reduces the risk of severe GVHD without compromising its efficacy has markedly reduced the toxicity of DLI. The explanation of why early DLI is associated with such a high risk of severe GVHD is unclear, but may relate to more rapid expansion of donor alloreactive T cells in the profoundly lymphopenic environment present in the first few months after transplantation or the residual inflammatory effects of conditioning-induced injury. An alternative approach in patients who relapse late (usually more than 12–18 months post-transplant) is a second allograft using an RIC regimen. Although associated with an increased TRM, a number of studies now show a significant chance of long-term disease-free survival after a second allograft in a significant minority of such patients. Finally, it is increasingly likely that newer approaches such as CAR-T therapy will be employed to salvage patients in certain disease groups. Wherever possible, patients should be offered the option of entering clinical trials.

Future developments in stem cell transplantation

Autologous SCT

Relapse remains the commonest cause of treatment failure in patients after autografting. Most of the drugs contained in preparative regimens are already administered at close to the limit of extramedullary toxicity and there is therefore little room for further dose escalation. Consequently, efforts to reduce relapse rate will depend on either altering the mode of delivery of currently used agents or the development of new forms of chemotherapy or radiotherapy. Many haematological malignancies are highly radiosensitive and there is interest in the possibility of increasing the effective dose of irradiation delivered to the marrow without increasing organ toxicity using radiolabelled immunoconjugates. Early-phase clinical studies using α (^{131}I) or β (^{90}Y) emitters conjugated to haemopoietic antigens, such as CD45 or CD66, demonstrate that such an approach is feasible, and phase II studies in myeloma and lymphoma are ongoing. An alternative strategy is to utilise adjunctive CAR-T cell therapy in patients who relapse after an autograft for myeloma or lymphoma and additional research concerning sequencing of transplant and CAR-T cell therapy is required.

Allogeneic SCT

Major advances in allogeneic transplantation in the past decade include: (i) the introduction of novel GVHD preventative strategies (e.g. PTCy, abatacept) to permit HLA-mismatched transplantation; (ii) the effective use of new drugs to salvage patients with steroid-refractory acute (ruxolitinib) and chronic GVHD (ruxolitinib, belumosudil); (iii) the use of donor CAR-T cells in selected patients who relapse following allo-SCT. There are now a plethora of early phase clinical trials in the arena of GVHD and GVL. In the future, the concept of GVL may become uncoupled from allogenic transplant, and instead used to refer to the use of any donor or 3rd party cell designed to target leukaemia selectively; these therapies will need to provide the breadth and depth of classical GVL while avoiding on- or off-target toxicities.

The breakthrough success of ruxolitinib treatment for GVHD stands as testament to the value of pre-clinical research being translated into clinical trials. It is likely that this drug will be tested in preventative or first-line therapy, alone or in combination with other drugs. In acute GVHD, recombinant IL-22 has been tested in a clinical trial for its capacity to improve epithelial regeneration in the gut. Newer agents such as Hedgehog pathway inhibitors, anti-CSF-1 antibody and pirfenidone are being evaluated for their potential anti-fibrotic actions in chronic GVHD. In the setting of TCD, where the risk of GVHD is low, the emphasis is on minimising the impairment in immune reconstitution and increased infectious complications caused by depletion of donor T cells. One promising approach by which this might be achieved is through the use of 'addbacks' of antigen-specific T cells recognising common post-transplant viral pathogens such as CMV, EBV or adenovirus. Such cells can be generated either by coculturing donor lymphocytes with APCs primed with candidate antigens, followed by expansion, or by early selection on the basis of interferon-γ secretion (a technique termed 'IFN-γ capture'). Alternatively, antigen-specific cells might be selected directly from peripheral blood using immunomagnetic techniques based on labelling of antigen-specific cells using HLA class I–peptide multimers. In order to improve coverage against multiple pathogens, infusion of donor lymphocytes from which alloreactive T cells have been depleted may hasten immune reconstitution without incurring the risk of GVHD normally associated with DLI. 'Allodepletion' strategies being studies include directly removing donor CD8$^+$ T cells from the infusion, *ex vivo* depletion of alloreactive T cells (using immunotoxin against activation markers) following stimulation by recipient APCs, or removal of naïve (CD45RA$^+$) T cells from the graft. There is also substantial interest in the feasibility of transferring naturally occurring or *ex vivo* expanded CD4$^+$ FoxP3$^+$ or IL-10-producing regulatory T cells as

a means of preventing conventional T cells from inducing GVHD. The best approach for isolating and expanding these regulatory populations remains unknown, but the results from early trials have shown promise. At the time of writing, prospective randomised clinical trials have commenced to test the efficacy of CD45RA-depleted grafts or the addback of directly isolated, donor-derived regulatory T cells.

From first principles, reducing the risk of relapse can be achieved by increasing the intensity of the conditioning regimen (ideally without increasing transplant toxicity), administering anti-leukaemic therapies in the post-transplant period or by optimising the GVL effect. Donor T cells that have been expanded selectively to recognise tumour-associated antigens or lineage-restricted minor antigens (e.g. by expanding donor cells in the presence of peptide mixes) may prove effective and have shown feasibility in several trials. Perhaps the most exciting approach under investigation is the gene engineering of T cells to express T cell receptors (TCRs) or CARs specific for tumour-associated antigens. *TCR* gene therapy has the advantage of targeting tumour antigens derived from intracellular proteins but is limited by the need to use receptors that are restricted to particular HLA types, and also by the potential for cross-reactivity with other antigens. Proof-of-concept has already been shown for T cells transduced with a TCR reactive against a tumour-associated antigen (WT-1) in patients with high-risk AML. It is highly likely that the repertoire of TCR available to treat patients will increase rapidly over the next few years. Emerging evidence also supports the use of CAR-T cells in selected patients; these have the advantage that they are not HLA-restricted. For both TCR- and CAR-T therapies, gene editing to remove endogeneous *TCR* and *HLA* genes, raises the prospect of 'off the shelf' therapies from universal donors.

The challenge will be how such therapies can progress from first-in-human trials to prospective, controlled trials that evaluate efficacy and lead to regulatory approval. The complexity and cost of newer therapies, coupled with a high attrition rate when tested at phase I/II level means that most experimental therapies are unlikely to be incorporated into large randomised prospective trials; this could act as a powerful disincentive to the biopharmaceutical industry investment. There is therefore an important need to create collaborative groups including industry that seek to evaluate multiple therapies and to use adaptive trial designs to select winning strategies.

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CHAPTER 38

Clonal haematopoiesis

38

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Introduction

'… if variations useful to any organic being do occur, assuredly individuals thus characterised will have the best chance of being preserved in the struggle for life; and from the strong principle of inheritance they will tend to produce offspring similarly characterised. This principle of preservation, I have called, for the sake of brevity, Natural Selection'. While Charles Darwin set out this theory of evolution in relation to the adaptation of biological organisms over generations, the same fundamental principles apply to the microcosm in which haematopoietic stem cells (HSCs) operate over the lifetime of a single individual.

From the moment a human zygote undergoes its first cell division, its genetic material is susceptible to alteration, such that the DNA sequences of its two daughter cells might not be identical. This process of somatic mutation acquisition continues inexorably through life in all tissues, including in the haematopoietic system. In young individuals, the absolute number of mutations in HSCs is small; diversity in the pool is therefore low and these relatively homogeneous stem cells exist in a rich bone marrow environment. In this setting, the forces of Darwinian selection are weak and many or all of the 50–200,000 HSCs will be equally adept at self-renewal and production of mature blood cell progeny. In contrast, by old age, the passage of time has allowed substantial accumulation of somatic mutations – it is estimated that a typical 70-year-old human harbours up to 70 mutations per protein-coding gene across the HSC population – giving rise to considerable diversity in the stem cell pool. These conditions are ideal for strong natural selection, with functionally variable HSCs now existing in a bone marrow niche altered by ageing. In line with Darwin's theory of evolution by natural selection, the HSCs best suited to the altered niche 'will have the best chance of being preserved in the struggle for life', and their cognate clones will expand relative to those less fit. This relative fitness advantage and subsequent expansion is the defining feature of clonal haematopoiesis (CH).

A historical perspective

Blood cancers are the prototypical examples of CH, with their clonal nature first demonstrated by karyotypic analyses in the 1960s. However, it would be a further two decades before CH was identified in individuals without blood cancer, when skewed X chromosome inactivation patterns were observed in elderly females. In normal embryonic development, one of the two X chromosomes is randomly inactivated in every cell, with approximately half of all early embryonic cells inactivating the paternal X chromosome, and the other half inactivating the maternal X chromosome. Since each cell's X inactivation state is stably maintained across cell divisions, the same balanced allelic ratios of X inactivation might be expected in the HSC pool and circulating progeny. However, investigators did not consistently observe such balance and, instead, some females, particularly older females, displayed skewed X inactivation ratios in blood cells. It was concluded that such skewing resulted from an enlarged clone arising from a single HSC, with consequent overrepresentation of this HSC's X chromosome inactivation pattern (XCIP) in sampled blood cells (Figure 38.1).

Harnessing advances in next-generation sequencing, a series of landmark studies from 2009 onwards steadily characterised

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).

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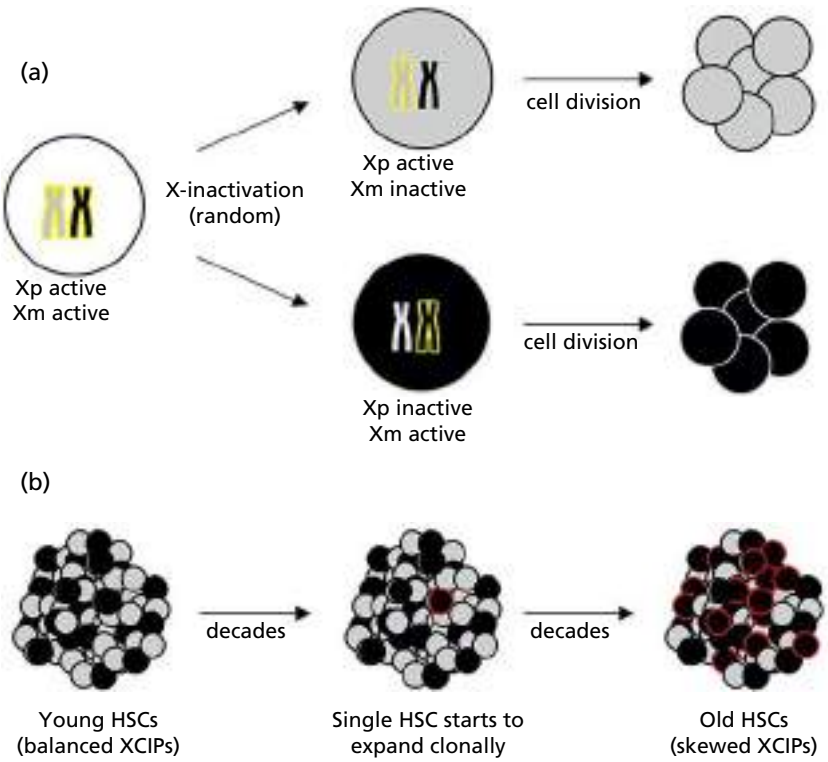

Figure 38.1 Detection of clonality through X-chromosome inactivation patterns (XCIPs). (a) X-inactivation is an epigenetic process that switches off gene expression in one of the two X chromosomes in every cell of the early female embryo. The inactivated X chromosome remains silenced in all cells that descend from the original embryonic cell within which X-inactivation took place. (b) As the process of X-inactivation is random, the ratio of cells bearing an inactive maternal X chromosome versus an inactive paternal X chromosome is expected to be balanced (balanced XCIPs). However, if a single HSC expands clonally *(red circled cell),* the average XCIP in the pool will be skewed towards the pattern of the clonal HSC and its progeny. Xm, maternal X chromosome; Xp, paternal X chromosome; ●, cell with inactive Xp; ●, cell with inactive Xm.

the somatic mutations driving myeloid malignancies. The question that followed was whether these same mutations could drive clonal expansions in individuals without myeloid malignancies. The first affirmation of this emerged in 2012, when mutations in *TET2* (a gene coding for an enzyme that oxidises methylated DNA) were detected in elderly females with skewed XCIPs. Two years later, several large-scale studies that collectively analysed exome or targeted gene sequencing of blood DNA from over 35,000 individuals, verified the phenomenon of CH driven by leukaemia-associated somatic mutations, revealing that it increased in prevalence with advancing age, particularly after the 6th decade of life. Notably, more than 95% of mutations occurred in just 8 driver genes, coding for enzymes involved in DNA methylation (*DNMT3A* and *TET2*) and chromatin regulation (*ASXL1*), proteins involved in cellular growth signalling (*JAK2*) and the DNA damage response (*TP53*, *PPM1D*), and splicing factors (*SF3B1*, *SRSF2*) (Figure 38.2).

Subsequent studies began to uncover additional fundamental characteristics of age-related haematopoietic clonal expansions. Firstly, while the close relationship between age and CH was consistently observed, estimates of the absolute prevalence depended heavily on the sensitivity of the sequencing assay utilised. Indeed, with more sensitive techniques, clonal mutations could be identified in almost all older individuals (Figure 38.3). Secondly, it was noted that expanded blood cell clones did not always harbour recognisable driver mutations and the cause of clonal expansion in these cases could not be determined. Thirdly, some studies identified larger chromosomal alterations as drivers of CH, such as loss of chromosome Y, trisomy 8 and deletions of the long arms of chromosomes 5 and 20. However, these mosaic chromosomal alterations (mCAs) were not as common as clonal haematopoiesis associated with single gene mutations.

Defining clonal haematopoiesis

CH is a clonal phenomenon arising from a multipotent HSC, distinguishing it from other precursor conditions such as monoclonal gammopathy of undetermined significance (MGUS) and monoclonal B-cell lymphocytosis (MBL), which originate

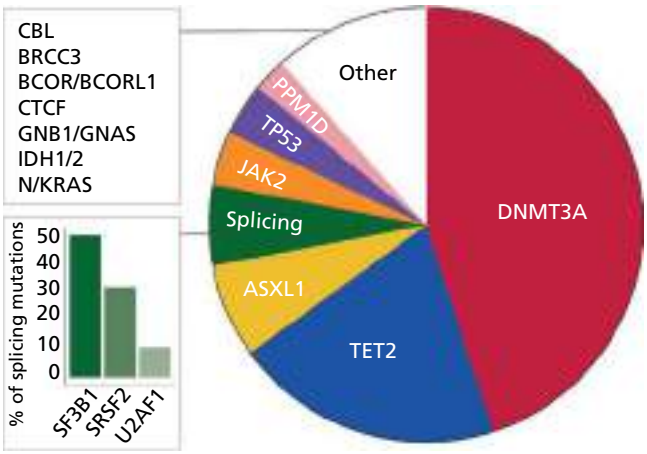

Figure 38.2 Relative prevalence of mutations in different CH driver genes. The proportions depicted here are approximate and derived from large studies of individuals with an average age of ~60 years. Proportions vary between studies, due mainly to differences in age distribution, but also to sequencing technology/depth of coverage. Driver genes in the 'Other' segment are much less commonly found in CH than the individually labelled genes on the pie chart. The list of 'Other' driver genes given in the adjacent box is not exhaustive but includes the more commonly mutated genes in this group.

in lymphoid progenitors. However, the precise definition of CH remains a matter of debate and a series of related descriptors have been coined (Table 38.1). When cytopenias are present, the clinical terms 'idiopathic cytopenia of undetermined significance' (ICUS) and 'clonal cytopenia of undetermined significance' (CCUS) should be used, provided that bone marrow examination is normal, or shows mild abnormalities not sufficient to meet the diagnostic criteria for a myeloid malignancy. These terms are defined in Table 38.1.

The term 'CHIP', referring to CH with driver mutation(s) at a variant allele fraction (VAF) of 2% or greater (corresponding to 4% of cells for heterozygous mutations), has been proposed as a useful category for CH associated with an increased risk of clinical sequelae, such as an increased risk of cardiovascular disease or progression to myeloid neoplasia. This is probably an oversimplification, as smaller clones driven by 'high-risk' genes might also be important to identify. However, since ultrasensitive sequencing can identify tiny CH clones in almost everyone (Figure 38.3b), a pragmatic cut-off based on clone size is useful for inferring likely clinical relevance. In practice, the term 'CHIP' can serve this purpose.

Mechanisms underlying clonal expansion

The process of HSC division results in two daughter cells that follow one of three possible fates: (i) one HSC and one differentiated progenitor, (ii) two differentiated progenitors, or (iii) two HSCs.

Table 38.1 Definitions of CH and related terms.

Term	Description
Clonal Haematopoiesis (CH)	The presence of an expanded clone of blood cells arising from a single HSC.
Clonal Haematopoiesis with a putative driver (CH-PD)*	The presence of an expanded clone of blood cells with an identifiable somatic driver mutation(s), in the absence of blood cytopenias.
Clonal Haematopoiesis with unknown driver (CH-UD)	The presence of an expanded clone of blood cells without an identifiable somatic driver, in the absence of blood cytopenias.
Clonal haematopoiesis of indeterminate potential (CHIP)	The presence of an expanded clone of blood cells with an identifiable somatic driver mutation(s) at a variant allele fraction (VAF) of ⩾2%, in the absence of blood cytopenias.
Clonal cytopenia of undetermined significance (CCUS)	Persistent cytopenia(s) that is not explained by haematologic or non-haematologic conditions and does not meet diagnostic criteria for a myeloid neoplasm, in the presence of CH.
Idiopathic cytopenia of unknown significance (ICUS)	Persistent cytopenia(s) that is not explained by haematologic or non-haematologic conditions and does not meet diagnostic criteria for a myeloid neoplasm, in the absence of CH.

*The putative driver can be a mutation in a single gene or a chromosomal aberration (the latter is also known as 'mosaic chromosomal abnormality').

The first pattern is the most common and maintains a stable number of HSCs, while enabling haematopoiesis to continue. The second pattern leads to HSC loss. The third pattern – symmetric division resulting in two clonal HSCs – leads to HSC gain. An increased propensity to this third fate is a property known as *increased self-renewal*, which is the core characteristic of the persistent clonal expansion defining CH. A sustained increase in self-renewal leads to a steady increase in the number of clonally-derived HSCs (Figure 38.4).

While enhanced self-renewal is a prerequisite for CH, the factors promoting this phenotypic change are complex and can be both *intrinsic* and *extrinsic* to HSCs, with intricate interplay between the two. Ageing is a pervasive factor that impacts both HSCs themselves and the environment in which they reside and is closely linked with the development of CH. The mere passage of time allows for somatic mutations to be acquired and for mutant clones to expand to detectable levels. It is notable that

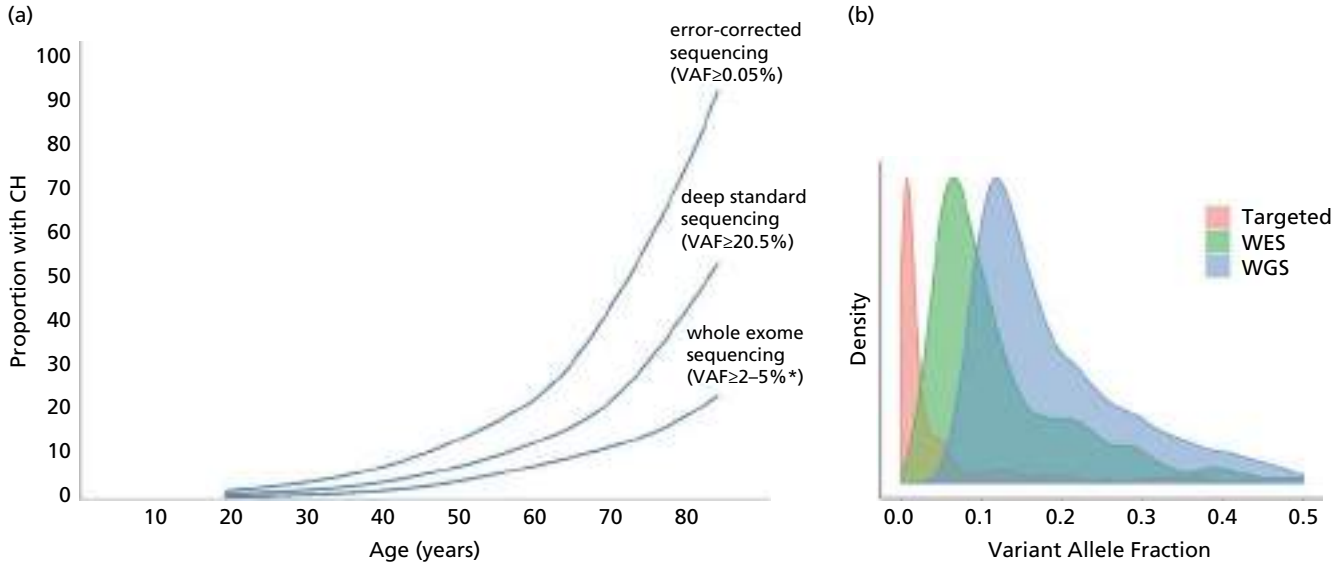

Figure 38.3 CH prevalence and detection in the general population. (a) Different estimates of the age-related prevalence of CH in the general population, according to the sensitivity of the sequencing technology used. *The sensitivity of whole-exome sequencing varies by driver gene due to uneven sequencing depths for different exons/genes. (b) Scaled density plots for Variant Allele Fraction (VAF) of driver mutations detected using different sequencing methods, in descending order of depth of coverage: Targeted sequencing (Abelson *et al*. Nature 2018), Whole exome sequencing (WES) (Jaiswal *et al*. NEJM 2014) and Whole Genome Sequencing (WGS) (Bick *et al*. Nature 2020). This demonstrates that smaller clones (with lower VAFs) can be detected when the depth of sequencing is higher.

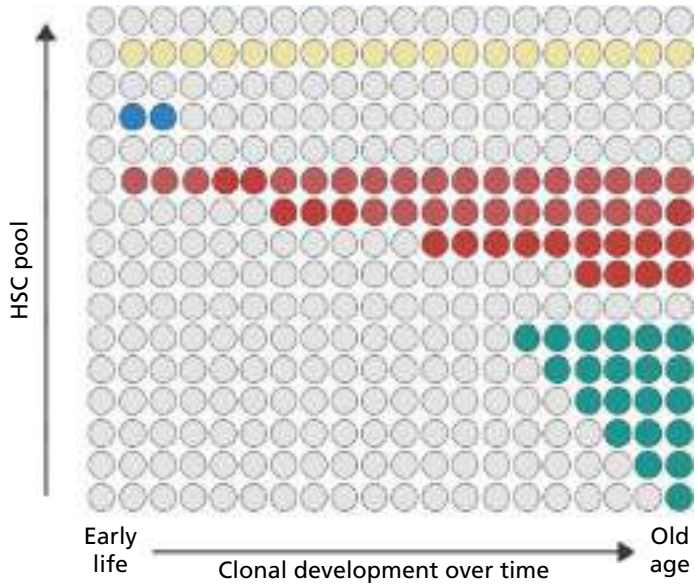

Figure 38.4 Clonal selection and its influence on HSC composition over life. In this schematic, an HSC is represented by a circle, the colour of which reflects its particular mutational status: grey cells carry no somatic driver mutations and other colours depict cells with different mutations. In early life, few somatic mutations have been acquired – in this example, none that drive clonal expansion – and the polyclonal HSC pool is relatively uniform. Over time, HSCs accumulate somatic mutations, which can impact fitness in a number of different ways: (i) Neutral (yellow) – these mutations do not alter fitness and there is no clonal expansion; (ii) Deleterious (blue) – these mutations diminish fitness and affected HSCs are removed from the pool; (iii) Advantageous – these mutations can confer a subtle advantage (red), in which case clonal expansion is slow and might only become evident many decades after mutation acquisition, or stronger advantage (green), when expansion is more rapid. It is important to note that, even amongst mutations that confer a fitness advantage, fixation of the mutant HSC clone in the pool and subsequent expansion is not inevitable, and many clones die out; a clone's chance of persistence is proportional to its fitness. The lifelong process of selection and clonal expansion is universal, and haematopoiesis inevitably becomes oligoclonal by advanced old age.

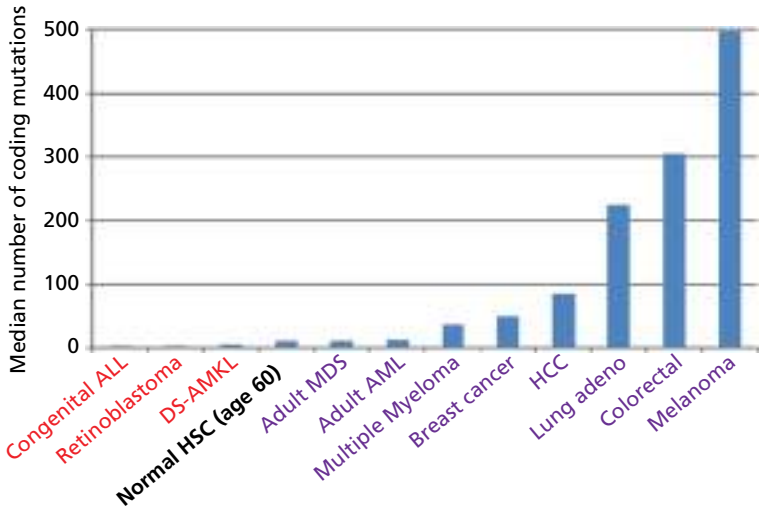

Figure 38.5 Median number of coding mutations in normal-aged HSCs and in different cancer types. In the *x*-axis labelling, paediatric cancers are coloured red, adult cancers are coloured purple and normal HSCs from individuals aged ~60 are in bold type. Notably, the average number of coding mutations in normal-aged HSCs (10) is similar to that in adult AML (10) and MDS (13), and considerably lower than many common adult cancer types. ALL, Acute lymphoblastic leukaemia; DS-AMKL, Down Syndrome-Acute Megakaryoblastic Leukaemia; HSC, haematopoietic stem cell; MDS, Myelodysplastic Syndrome; AML, Acute Myeloid Leukaemia; HCC, Hepatocellular carcinoma.

the number and type of somatic mutations found in an expanded CH clone are similar to those found both in normal-aged HSCs and in myeloid malignancies, indicating that increased mutation rate is not usually a factor in the aetiology of these clonal disorders. This is unlike most of the common adult cancers, where specific mutagens causing larger numbers of mutations are an important factor in disease pathogenesis (Figure 38.5).

However, the influence of ageing on the development of CH goes beyond its simple association with time and mutation acquisition. Most significantly, aged HSCs display cell-intrinsic defects, with altered transcriptional and epigenetic landscapes suggestive of a molecular hardwiring of the ageing process, as well as defects in DNA repair and polarity. These cell-autonomous processes can also be influenced by extrinsic signals and, importantly, the bone marrow microenvironment/niche changes significantly with ageing. Amongst other changes, adipogenesis is favoured over osteogenesis, there is a decline in specialised vascular niches, and the chemical milieu is increasingly inflammatory. In this setting of progressive degeneration of HSCs and their environment, those HSCs that self-renew most effectively will expand relative to their peers, leading to CH. The advantage is often attributable to an identifiable HSC driver mutation, but recent advances suggest that such drivers are not always detected. Apart from ageing, other factors impact clonal expansion more specifically through either cell-intrinsic or cell-extrinsic processes, and these are considered separately below.

Cell-intrinsic contribution to HSC fitness

The genes recurrently mutated in CH broadly fall into three main classes: epigenetic regulators (*DNMT3A*, *TET2*, *ASXL1*), splicing factors (*SF3B1*, *SRSF2*, *U2AF1*) and DNA damage response (DDR) genes (*TP53*, *PPM1D*), with rarer contributions from other epigenetic genes, signalling molecules and haematopoietic transcription factors (e.g. Figure 38.2). An improved understanding of the function of the affected genes can give insights into their role in CH. However, developing appropriate experimental models can be challenging, with the molecular effects of driver mutations often imparting only subtle advantages on host HSCs such that expanding CH clones take decades to become detectable. In addition, any such advantage might only be realised when a mutant HSC resides in a particular environment or context, which might be difficult to recapitulate in the laboratory. Nevertheless, some progress has been made in dissecting the mechanistic role these genes play in driving clonal expansion.

Epigenetic regulators: *DNMT3A* and *TET2*

DNA methylation plays a key role in gene regulation and cellular identity/behaviour, with methylation, particularly at promoters, generally considered a repressive mark. Central to this regulatory process are the enzymes responsible for adding methylation marks, DNA methyltransferases (DNMTs), and the ten-eleven translocation (TET) family, which can remove these marks. While a number of DNMT and TET family members can catalyse these reactions, *DNMT3A* and *TET2* are the genes most commonly mutated in CH and haematological malignancies. Overall, loss of *DNMT3A* function is associated with DNA hypomethylation, while loss of *TET2* function is associated with DNA hypermethylation. Mechanistic studies discussed below have shed some light on the consequences of mutations in these genes, but the question of why they lead to clonal expansion remains largely answered.

Truncating *DNMT3A* mutations occur across the length of the gene, with missense mutations clustering in known structural and functional domains. Importantly, the relative frequency of different DNMT3A mutations in CH is distinct from that observed in myeloid neoplasms (particularly AML), which much more frequently affect the R882 hotspot residue. The distribution of mutations in *TET2* is similar, with missense mutations concentrated in the two conserved regions, and truncating

mutations spread uniformly across the entire gene. However, unlike *DNMT3A*, there are no major mutation hotspots in *TET2*. Mutations in *DNMT3A* and *TET2* are usually heterozygous and lead to loss of protein function. Mouse models carrying loss-of-function mutations in either of these genes display a propensity to develop myeloid malignancies, while their HSCs have a self-renewal advantage over wild-type HSCs, as exemplified by an increased ability to serially form colonies *in vitro* and an ability to outgrow wild type HSCs in competitive transplantation assays *in vivo*.

Why loss of function of either *TET2* or *DNMT3A* has such a profound impact on HSC biology remains elusive. Different mechanisms have been suggested, such as promoter hyper- or hypomethylation leading to changes in the expression of genes that regulate stem cell self-renewal, senescence or proliferation. However, the identity of these genes remains elusive despite extensive research efforts. For example, in both human and mouse cells with *DNMT3A* or *TET2* mutations, there are no marked or consistent gene expression changes and a limited overall correlation between alterations in DNA methylation and changes in expression levels of differentially methylated genes. In addition, these enzymes have complex interactions with other epigenetic regulators, their associated methylation changes might influence 3D genome interactions, and they might play roles independent of methylation. Especially perplexing is the fact that *TET2* and *DNMT3A* have ostensibly opposite biochemical functions, yet mutations in both result in similar phenotypes of enhanced self-renewal, a major conundrum in the field.

Recent longitudinal studies designed to capture the lifelong clonal dynamics of CH have revealed further subtleties. While *TET2* mutations seem able to initiate clonal expansion at any age and exhibit similar growth patterns across life, *DNMT3A*-mutant clones tend to expand faster earlier in life, before decelerating in older age. Why mutations in these genes are associated with different clonal behaviours across life is currently unknown, but the observation is important nevertheless. *Firstly*, it highlights the fact that the ability of these mutations to drive clonal expansion is intimately tied to the cellular and environmental context in which they exist (discussed in more detail later in this chapter). *Secondly*, the disparity in behaviour likely explains why *DNMT3A* is the dominant CH driver gene through the first 6-7 decades of life, only to be 'overtaken' by *TET2* in people aged 75 years or older (Figure 38.6).

Splicing factors: *SF3B1*, *SRSF2* and *U2AF1*

Precursor messenger RNA splicing is a critical step in the post-transcriptional regulation of gene expression, providing substantial expansion of the functional proteome of eukaryotic organisms in relation to the number of genes. The process is executed by the spliceosome, a large and highly dynamic ribonucleoprotein complex. Alternative splicing patterns are tightly regulated but are fluid, changing for example over each 24-hour cycle with the mammalian circadian rhythm, and over a lifetime with advancing age.

Disruption of alternative splicing can be observed in many tumour types, often mediated by mutations in components of the spliceosome. Spliceosome gene mutations are prevalent in myeloid cancers and in CH, and most commonly affect three genes controlling 3′ splicing: *SF3B1*, *SRSF2* and *U2AF1*. Mutations in each of these occur as heterozygous point mutations at specific residues, suggesting gain-of-function alterations. Indeed, mutant *SF3B1* promotes usage of alternative branch points, leading to usage of cryptic 3′ splice sites, and *SRSF2*/*U2AF1* mutations are characterised by alternative exon usage. In addition, mutations in spliceosome genes are generally mutually exclusive with one another, presumably due to redundant effects and/or a limit on cellular tolerance of disrupted spliceosome function. While these biochemical abnormalities lead to aberrant splicing of hundreds of genes, the exact mechanisms by which these mutations lead to clonal expansion remain largely unknown. Extensive analysis has revealed very little overlap between the aberrantly spliced

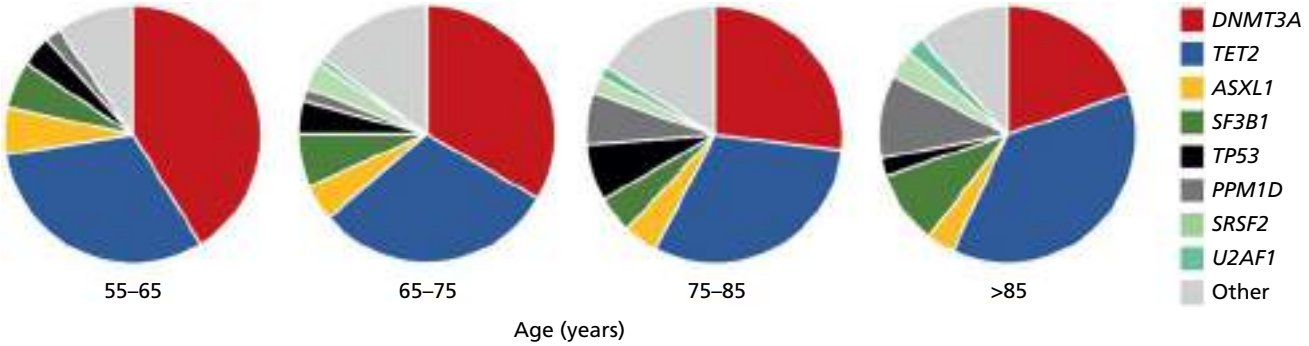

Figure 38.6 Changing spectra of CH-driving mutations with advancing age. *DNMT3A* mutations dominate the landscape of CH in people younger than 60 years old. With advancing age beyond 60 years, *DNMT3A* mutations steadily lose this dominance as *TET2* mutations continue to rise inexorably to become the most common CH drivers beyond the age of ~80 years. Similarly, mutations in splicing factor genes and *PPM1D* become more common in advanced old age (Source: Data from Fabre *et al.* Nature 2022).

target genes associated with different splicing mutations, and the mis-spliced genes are generally not those known to drive cancer. This suggests that the clonal advantage imparted by these mutations might not be explained by any single mis-splicing event, but instead that multiple splicing alterations might cooperatively contribute to clonal expansion. Alternatively, other unknown effects of splicing gene mutations might operate.

The observation that HSCs from mice mutant for *Sf3b1*, *Srsf2* or *U2af1* have a competitive disadvantage *in vivo*, unlike the clonal expansion observed in humans with these mutations, adds further intrigue to the biology. The disparity might be explained by differences in alternative splicing patterns in humans and mice, in part because the intronic sequences identified by mutant splice factors are not well conserved between mice and humans. Another possibility is that splicing gene mutations only drive clonal expansion in a particular context, which could relate to conditions within the mutated HSC or to the environment in which the HSC resides. This possibility is supported by studies showing that splicing gene mutations do not initiate clonal expansion in young individuals and only do so in later life, when they can drive rapid growth. This implies an intimate relationship between these mutations and ageing, but the nature and biology of this interaction remain elusive.

Additional classes of driver mutation

For some other mutant genes, the mechanisms underlying clonal expansion are better understood. For example, activating mutations in *JAK2* that drive 3–4% of CH in most studies, lead to constitutive signalling through growth factor receptors, leading to clonal expansion. In contrast, a number of genes involved in the DNA damage repair (*DDR*) are recurrently mutated in CH, notably *TP53*, *PPM1D* and less often *ATM* and *BRCC3*. *TP53* is a key tumour suppressor in humans and CH driver mutations lead to loss-of-function. *PPM1D* is a negative regulator of the DDR; driver mutations occur almost exclusively in the terminal exon of the gene, truncating the protein to generate a highly stabilised form that attenuates the entire *DDR* (i.e. an effect similar to that of *TP53* loss). *DDR* mutations impart HSCs with a clonal advantage relative to wild-type HSCs, particularly when an individual is exposed to DNA-damaging agents, such as cytotoxic chemotherapy. However, these mutations drive CH quite commonly, including in individuals without known prior exposure to any cytotoxic agent, suggesting that they might confer advantage through additional mechanisms, or that endogenous or agnogenic DNA damage may also exert a selective pressure.

Other drivers of CH remain less extensively studied, and their biology is poorly understood. *ASXL1*, for example, the third most common *CH* driver gene, is a member of the polycomb repressive complex 2 (*PRC2*), which plays a key role in stem cell biology and *Hox* gene regulation. Driver mutations in this gene are always truncating and occur exclusively in the terminal two exons (11 and 12), but the precise way in which the truncated protein leads to clonal advantage is unknown.

In addition to the more recurrently mutated genes driving CH, there is a long tail of less common drivers, including cohesin genes such as *RAD21*, *SMC3* and *STAG2*, G-protein genes such as *GNAS* and *GNB1* and epigenetic regulators such as *BCOR*, *BCORL1*. In fact, it is almost certain that this 'tail' of drivers extends well beyond what has been characterised, with mutations in many other genes conferring a clonal growth advantage which, over decades, can lead to clonal expansion, yet mutations in individual genes are not sufficiently recurrent to be identified in cohorts of the sizes studied to date. This concept is substantiated by studies that used passenger mutations to identify clonal expansions in peripheral blood, in the absence of any identifiable CH drivers. Furthermore, reconstructions of haematopoietic phylogenies of elderly individuals using whole-genome sequencing of single-cell-derived colonies, revealed that the presence of so-called 'unknown-driver' expansions is the norm in old age, with evidence that such expansions have grown under positive selection rather than due to neutral drift.

Finally, somatic acquisition of large chromosomal alterations has also been found to drive CH in some individuals. The commonest such genetic lesion seen in CH is mosaic loss of the Y chromosome (mLOY), with more than 40% of elderly men harbouring clones driven by LOY. Other less common mCAs are also observed and are broadly the same as those found in haematological cancers, such as deletions of 5q, 20q and 17p.

Inherited factors

Germline variation influences the risk of developing CH, the identity of the CH driver mutation and the rate of clonal expansion. A heritable component was initially implied by epidemiological observations, with early reports of CH being more common in men and less common in individuals identifying as Hispanic or East Asian. Initial cohort studies analysing concordance for CH within identical twin pairs did not find a strong inherited predisposition, but more recently genome-wide association studies have revealed a number of risk loci. One set of risk alleles is associated both with CH and with neoplasia in multiple tissues, via dysfunction of genes that maintain genome integrity (e.g. *TERT*, *CHEK2*, *ATM* and *PARP1*). A second set of germline loci is associated with the acquisition of mutations in specific driver genes; for example, the *JAK2* 46/1 haplotype predisposes to the *JAK2* p.V617F mutation via a *cis*-haplotype effect. A third set of risk loci, including, *TCL1A, CD164* and *SMC4*, are linked specifically to CH development, rather than neoplasia more generally. *TCL1A* is particularly interesting because polymorphisms associated with higher *TCL1A* mRNA expression are associated with CH due to mutations in most of the common driver mutations, including *TET2*, *ASXL1*, *SF3B1*, *SRSF2* and *JAK2*. In contrast, polymorphisms associated with low *TCL1A* expression increase the risk of *DNMT3A*-mutant

CH specifically. This reciprocal relationship of *TCL1A* polymorphisms with different CH subtypes suggests that its expression needs to be tightly regulated to prevent clonal expansion.

Characterising the landscape of inherited predisposition remains an active area of research and is important not only for quantifying an individual's risk of CH and its implications in the clinical setting but also for revealing fundamental aspects of the biology and mechanisms of clonal expansion.

Cell-extrinsic contribution to HSC fitness: environmental influences on clone selection

Simple calculations based on the number of HSCs, the size of the genome and the rate of acquisition of somatic mutations, as well as studies using exquisitely sensitive error-corrected sequencing, demonstrate that almost all individuals harbour HSCs with coding mutations in CH driver genes by middle age. However, only in a fraction of individuals do mutant clones expand to levels detectable by standard sequencing techniques. What determines whether such expansion occurs? The identity of the mutation and the timing of its acquisition are important determinants, but do additional extrinsic factors influence whether, or to what degree, a mutation with driver potential stimulates growth? Several lines of evidence suggest that such extrinsic factors do indeed play influential roles.

A clear indication that the environment can determine whether a mutation drives clonal growth or not, comes from the observation that the identities of 'driver' genes vary depending on the context, as illustrated in Figure 38.7. For example, in aplastic anaemia, where autoreactive T cells target and eliminate HSCs, clones with mutations that facilitate immune evasion selectively survive and expand relative to wild-type HSCs. In the very different scenario of genotoxic stress induced by cytotoxic chemotherapy, as discussed above, clones with mutations in DDR genes expand. As these new environments arise, previously neutral mutations now confer a growth advantage, implying that the mutations themselves are not inherently advantageous in isolation, but become so in the presence of particular extrinsic signals/contexts.

A role for non-mutation factors in clonal expansion is also suggested by longitudinal studies showing that clones in different

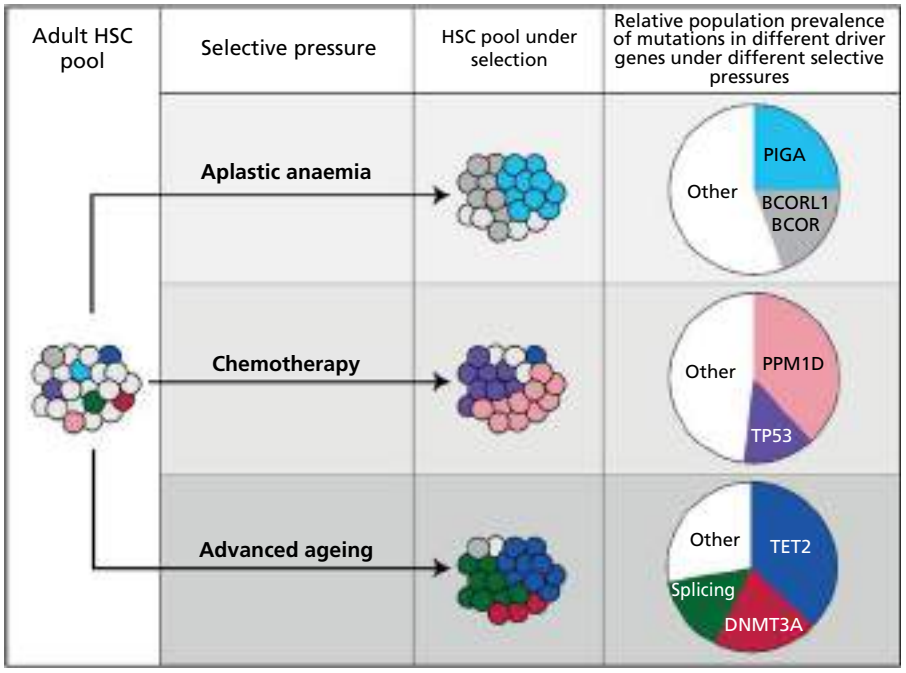

Figure 38.7 Driver gene spectra in different contexts. HSCs acquire somatic mutations inexorably over time, leading to a heterogeneous HSC pool represented by differently coloured cells at the left of the figure. Particular mutations confer a fitness advantage in specific contexts, leading to clonal selection and expansion. In aplastic anaemia (an autoimmune condition), mutations in genes such as *PIGA* allow an HSC to evade immune attack and expand relative to its peers. With exposure to chemotherapy, mutations in DNA-damage-response pathway genes (e.g. *TP53*, *PPM1D*) are selected. In advanced ageing, there is increasing selection for HSCs with mutations in splicing genes and in *TET2* (which 'overtakes' *DNMT3A*, also coloured in the figure for comparison, as the most common driver in this age group). Pie charts on the right of the figure illustrate the relative prevalence of mutations in different driver genes in different contexts.

individuals with identical mutations behave differently over time. Studies in allogeneic sibling transplantation have demonstrated that at least part of the explanation lies outside the mutant cell itself. Here, CH passed from donor to recipient, where the mutant HSC was identical (in all aspects, and not just in the identity of the driver mutation) but now housed in two independent individuals, was found to expand more rapidly in the recipient compared to the donor in most cases. This suggested that some aspect of the transplantation process or the milieu of the recipient bone marrow was particularly conducive to clonal expansion.

In the search for non-cell-autonomous factors influencing clonal growth, much interest has focussed on a potential role for inflammation, a feature of human ageing, particularly in relation to *TET2*-mutant CH. While the long-term function of normal HSCs is perturbed by sustained exposure to proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), *TET2*-mutant HSCs resist these signals and maintain functional integrity, providing them with a competitive advantage in a chronic inflammatory environment. Furthermore, *TET2* normally functions as a negative regulator of the inflammatory response in myeloid cells, in part by recruiting *HDAC2* to suppress IL-6 production. *TET2* loss-of-function mutations therefore potentially set up a positive feedback loop whereby the mutant myeloid cells secrete more IL-6, further augmenting their relative advantage over wild-type HSCs. Recent evidence suggests that the dependence of *TET2*-mutant clonal expansion on IL-6 inflammatory signals might be partly microbially-mediated, as it only occurred in mice with intestinal dysfunction and bacterial translocation, was reversed by antibiotic treatment and was absent in germ-free mice. Some evidence is also emerging that specific proinflammatory cytokines might induce expansion of CH with particular driver mutations, such as positive selection of *DNMT3A*-mutant clones by interferon-gamma signalling during chronic infection.

Finally, some studies have reported a weak association between smoking and CH, predominantly CH driven by *ASXL1* mutations. Furthermore, large genetic studies using Mendelian randomisation (an unbiased genetic approach for removing confounders) have suggested that smoking is actually a causative risk factor. While it is known that smoking does not induce mutations in HSCs, the reason for the link between smoking and CH remains unclear, although a chronic inflammatory state induced by smoking has been postulated.

Research into non-cell-autonomous influences on CH remains in its infancy but is one with translational potential. It is not currently possible to reverse somatic mutations in clonally expanded HSCs, but it is plausible that extrinsic signals could be altered therapeutically to minimise or negate any advantage conferred by the mutation.

Patterns and determinants of clonal behaviour through life

Pivotal to our understanding of CH, and a key starting point for elucidating underlying mechanisms, is characterising the natural history of clonal behaviour. Until recently, we had almost no insight into the timing of driver mutation acquisition, how/if this relates to the foundation of a mutant clone and how an established clone behaves over time. The first indication that CH driver mutations could be acquired very early in life (in fact, *in utero*), came from two independent studies of human twin pairs in 2020. Across both studies, the twins in each of 3 monozygotic pairs (out of a total of 161 pairs analysed) were found to harbour identical rare somatic driver mutations. It is extremely unlikely that each member of a pair acquired the same mutation independently and by chance. A more plausible explanation is that the somatic mutation occurred just once *in utero*, either prior to twinning or in an HSC whose progeny reached both twins through their shared circulation. The finding that the first step on the path to a clonal phenomenon associated with ageing can be initiated in the earliest stages of life is remarkable. A first glimpse into selection patterns and growth dynamics in CH came later the same year, when population genetic theory was applied to cross-sectional VAF data from ~50,000 individuals to infer the relative fitness of different driver mutations. While this broke new ground, revealing that positive selection, not neutral drift, is the major force shaping CH, the robustness of relative fitness calculations was limited by the study's reliance on two key assumptions: *first*, that a mutation's fitness is constant across time and, *second*, that the propensity for a mutation to found a clone is uniform through life.

These limitations were overcome when lifelong clonal dynamics were studied directly, by (i) tracking clones longitudinally through serial blood VAF measurements, and (ii) reconstructing haematopoietic phylogenies from whole-genome-sequenced single-cell-derived colonies to reconstruct growth trajectories. These analyses revealed that mutations in different driver genes induce characteristic patterns of clonal behaviour throughout life (Figure 38.8). For example, *DNMT3A*-mutant clones preferentially expand early in life and display slower growth in old age (~5% per year). By contrast, splicing gene mutations only initiate clones and drive expansion in old age, when growth is rapid (up to 50% per year for some *SRSF2*-mutant clones). *TET2* displays a more uniform behaviour throughout life, with mutant clones emerging and growing at similar rates (~10% per year) across all ages. Which factors underlie these disparate patterns of lifelong behaviour are unknown, but their elucidation will be critical to our understanding of CH. A further striking observation from longitudinal clonal tracking studies is that, by old age, almost all clones expand at a stable exponential rate over time.

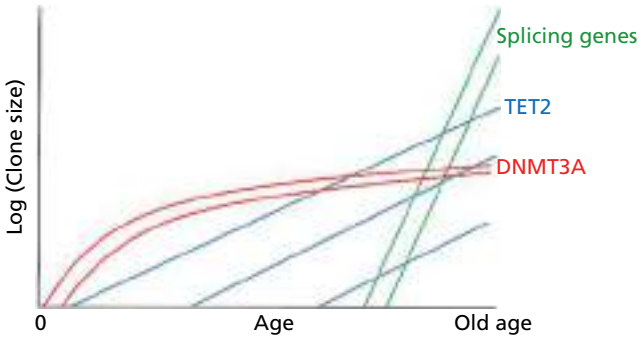

Figure 38.8 Lifelong driver-specific patterns of clonal growth. *DNMT3A*-mutant clones (depicted in red) emerge early in life and expand rapidly, before decelerating and growing slowly in old age. *TET2* mutations (blue) found clones fairly uniformly through life and drive more stable growth across ages. CH driven by splicing gene mutations (green) initiates almost exclusively late in life and exhibits very rapid expansion dynamics.

This constancy is compelling, not only from the biological perspective, but also due to its translational potential, with prediction of future clonal growth in the clinical setting now feasible.

Clonal haematopoiesis and myeloid malignancies

The association between CH and myeloid malignancies, including acute myeloid leukaemia (AML), myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPNs), is well documented and unsurprising given the shared mutational landscape. In this context, CH can be viewed as the first step towards malignancy, with the acquisition of additional cooperating mutations responsible for progression. For example, a well-documented pathway to AML is via *NPM1* and/or *FLT3* mutation acquisition in an expanded *DNMT3A*-mutant CH clone. However, co-mutation does not explain all malignant progression; for example, *SF3B1* is the sole identifiable driver gene in one-third of *SF3B1*-mutant MDS cases, and half of all *JAK2*-V617F-mutant MPNs harbour no additional drivers. In these cases, expansion of the single-mutant clone appears to be sufficient to cause disease.

Quantifying and dissecting the risk of malignant progression in people with CH has attracted substantial interest given its potential for use in future leukaemia early-detection/prevention strategies. Studies have shown that individuals at risk of progression to AML can be identified years in advance, as they have larger CH clones and/or harbour mutations in multiple genes or in high-risk genes such as *TP53, SRSF2, U2AF1, IDH1 and IDH2*. An integrated prognostic model incorporating multiple factors could identify approximately 40% of individuals at highest risk with a false positive rate of 1–2%. While helpful, this level of discriminatory power is not sufficient for use in population screening, as large numbers of individuals would be falsely categorised as high-risk. More recently, models have been developed for predicting an individual's risk of developing any myeloid neoplasia (CH risk score, CHRS) or each of the main subtypes (AML, MDS or MPN) separately (MN-Predict). These risk-prediction tools incorporate characteristics of the expanded clone, demographic features and routinely measured complete blood count and biochemical parameters, and quantify malignancy risk years into the future.

Clonal haematopoiesis and non-haematological disease

CH has the potential to lead to diverse organ dysfunction since: (i) clonal selection and function are uncoupled, such that HSCs gain advantage through enhanced self-renewal and survival, even when this adversely affects the quantity and quality of their differentiated progeny, (ii) HSCs are not spatially constrained, so clones can expand to constitute a very large fraction of haematopoiesis and (iii) HSCs give rise to many differentiated immune effector cells, which traffic to every organ/tissue. Indeed, CH is associated with increased mortality, explained predominantly by increased cardiovascular mortality. Several studies have quantified the excess mortality risk in individuals with sizeable CH clones (VAF≥2%) and found it to be 30–40% higher than in individuals without CH. In one study of primarily middle-aged individuals, the risk of coronary heart disease and ischaemic stroke was more than doubled by the presence of CH and was higher in individuals with larger mutant clones. This level of risk was comparable to that conferred by well-known contributors to cardiovascular diseases, such as high LDL-cholesterol levels, smoking and hypertension. Causality was demonstrated in mouse models, with the finding that abnormal pro-inflammatory *TET2*-mutant macrophages accelerated atherosclerosis in susceptible mice. Recent epidemiological evidence suggests that CH is also associated with congestive heart failure, and loss of *TET2* or *DNMT3A* led to worsening heart function in a mouse model of this disease, consistent again with a causal role. A causal link between CH and chronic liver disease has also been proposed (Wong, Embdin, Rogers et al, Nature 2023).

A recent study unexpectedly reported that CH is protective against Alzheimer's dementia and that the degree of protection scales with clone size. Mendelian randomisation analyses and microglial profiling suggested a causal link and it was proposed that mutant, marrow-derived cells might supplement a failing microglial system during ageing. It will be fascinating to see if these observations are confirmed in independent studies and to understand how CH interacts with other neurodegenerative disorders as this area of research broadens.

Other studies have postulated links between CH and other diseases of ageing, such as type 2 diabetes and chronic obstructive pulmonary disease, but causality has not been established. It is

possible that these associations are due either to CH representing a biomarker for advanced biological ageing, or to confounders such as smoking. Specific experimental models will be required for more definitive exploration of these relationships.

A key question is how CH mutations change the molecular biology of immune cells to effect influences on diverse disease types. The importance of *TET2* and *DNMT3A* in restraining inflammatory gene expression in innate immune cells is emerging, with a pro-inflammatory phenotype developing when function is perturbed. While the progressive low-level inflammation associated with ageing might be permissive to mutant clonal growth, it is also possible that CH contributes to this 'inflammaging', or, indeed, that both processes operate in parallel in a cycle of positive feedback. A role for CH genes in the adaptive immune response is also suggested, for example, by *DNMT3A* loss-of-function preventing CD8 T-cell exhaustion in the setting of persistent viral infection. It is also notable that stem cell transplant recipients with haematological cancers who receive donor HSCs from carriers of CH have higher rates of graft-versus-host disease and reduced relapse rates, perhaps due to stronger adaptive immune responses against both normal host tissues and tumour.

Clinical considerations and future perspectives

CH is common, indeed ubiquitous, by old age and is associated with an increased risk of blood cancers and excess mortality from non-malignant diseases. From a basic science perspective, the study of CH is providing fundamental insights into the interactions between ageing, somatic mutation and natural selection, processes that operate across all tissues throughout life. From a clinical perspective, the pervasiveness and implications of CH provoke several important questions and challenges.

First, how should we manage individuals found to have CH 'coincidentally' during investigations for minor or transient haematological abnormalities? In this context, it is reasonable to stratify such individuals by their risk of progression to myeloid malignancy, using predictive tools such as 'MN-Predict' or CHRS. Such tools can help identify higher-risk individuals for close monitoring or entry into clinical studies, taking into account other factors such as comorbidities and life expectancy. A second challenging area is the distinction between CH, CCUS and low-grade MDS, which should involve consideration of morphological findings in the context of the somatic mutations identified. It is possible that the details of classification will evolve as our understanding of the relationships between these clinical states improves. A third important question is how to manage patients found to have CH after chemotherapy or stem cell transplantation for solid or lymphoid cancers. In this context, CH due to high-risk mutations, such as those affecting *TP53*, is more common and probably more strongly associated with progression to secondary myeloid malignancy than sporadic *TP53*-mutant CH. Our ability to predict risk in this context is less developed, but some specialist hospitals are following such patients closely and developing expertise in their management, with specific clinical trials being considered. Another area of debate is the interpretation of persistent CH mutations after treatment for AML. Studies have shown that residual isolated *DNMT3A* mutations after AML therapy do not represent minimal residual disease (MRD) and do not carry a high risk of relapse. The implications of other persistent CH driver mutations, such as *TET2* or *ASXL1,* are less well understood; it is not known if they represent MRD when isolated, or whether they are associated with a higher risk of relapse or development of a new myeloid malignancy.

A final consideration is whether we should screen unselected individuals for CH at scale and, if so, how we should do this. While it is likely that screening will be implemented in some form in future, its utility is currently limited by the lack of therapeutic interventions to reduce or restrain CH. Blockade of downstream inflammatory molecules might be one way to manage associated disorders such as cardiovascular disease, but novel interventions will likely focus on suppressing mutant clones directly, with the potential to prevent progression to myeloid malignancy. Such approaches will likely be tailored to specific driver mutations but will require a much deeper understanding of precisely which factors govern the behaviour of particular mutant clones, and how this relates to myeloid disease. As risk stratification improves and potential therapies are developed, it will be imperative to test these in rigorous clinical trials, ideally in the setting of dedicated CH clinics. Successful implementation of genotype-specific interventions would represent the beginning of a new era of 'precision prevention', in which therapies are tailored to genetically-defined risk groups to prevent potential adverse sequelae.

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PART V

Haemostasis and thrombosis

CHAPTER 39

Normal haemostasis

39

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Introduction

Haemostasis is one of a number of protective processes that have evolved in order to maintain a stable physiology. It interacts with other body defence mechanisms, such as the immune system and the inflammatory response. These links are most clearly seen in ancient species such as the horseshoe crab (*Limulus polyphemus*), where a primitive 'coagulation' pathway is initiated by entry of endotoxin into the haemolymph. Vestiges of these links still exist in humans and may give rise to serious clinical consequences. For example, disseminated intravascular coagulation (DIC) can be initiated by Gram-negative septicaemia. Consequent upon the development of a high-pressure circulatory system, extra components have evolved and have resulted in a complex, highly integrated process in all vertebrates (Figure 39.1). Indeed, analysis of the haemostatic network in bony fish suggests that the network in its entirety evolved over 430 million years ago, prior to the divergence of bony fish from tetrapods.

The high blood pressure generated on the arterial side of the vertebrate circulation requires a powerful, almost instantaneous, but strictly localised procoagulant response in order to minimise blood loss from sites of vascular injury without compromising blood flow generally. Systemic anticoagulant and clot-dissolving components have also evolved to prevent extension of the procoagulant response beyond the vicinity of vascular injury, which would otherwise result in unwanted thrombus formation. The evolved haemostatic system is thus a complex mosaic of activating and inhibitory pathways that integrates its five major components (blood vessels, platelets, coagulation factors, coagulation inhibitors and fibrinolytic elements). In addition, the activated proteases of the coagulation system play an important role in cellular signalling, independent of fibrin generation. This occurs through activation of protease-activated receptors (PARs), a subfamily of seven-transmembrane G-protein-coupled receptors, leading to intra-cellular signalling that results in a plethora of cellular responses.

Overview of haemostasis

In the most simplistic terms, blood coagulation occurs when the enzyme thrombin is generated and proteolyses soluble plasma fibrinogen, forming the insoluble fibrin polymer, or clot. 'Haemostasis' refers more widely to the process whereby blood coagulation is initiated and terminated in a tightly regulated fashion, together with the removal (or fibrinolysis) of the clot as part of vascular remodelling. It is essentially the global process by which vascular integrity and patency are maintained over the whole organism, for its lifetime.

Although it is pedagogically convenient to present the subsystems of haemostasis as if they operate independently, the whole haemostatic mechanism is integrated *in vivo* so that thrombin generation is localised, limited and followed by fibrinolysis and tissue remodelling, which are also localised and limited. Furthermore, thrombin generation is not a simple exponential cascade, as was originally envisaged by Davie and Ratnoff when the 'waterfall' hypothesis was described in the 1950s (Figure 39.2). Rather, it is a complex network of interactions with positive and negative feedback loops. Thus, the effects of

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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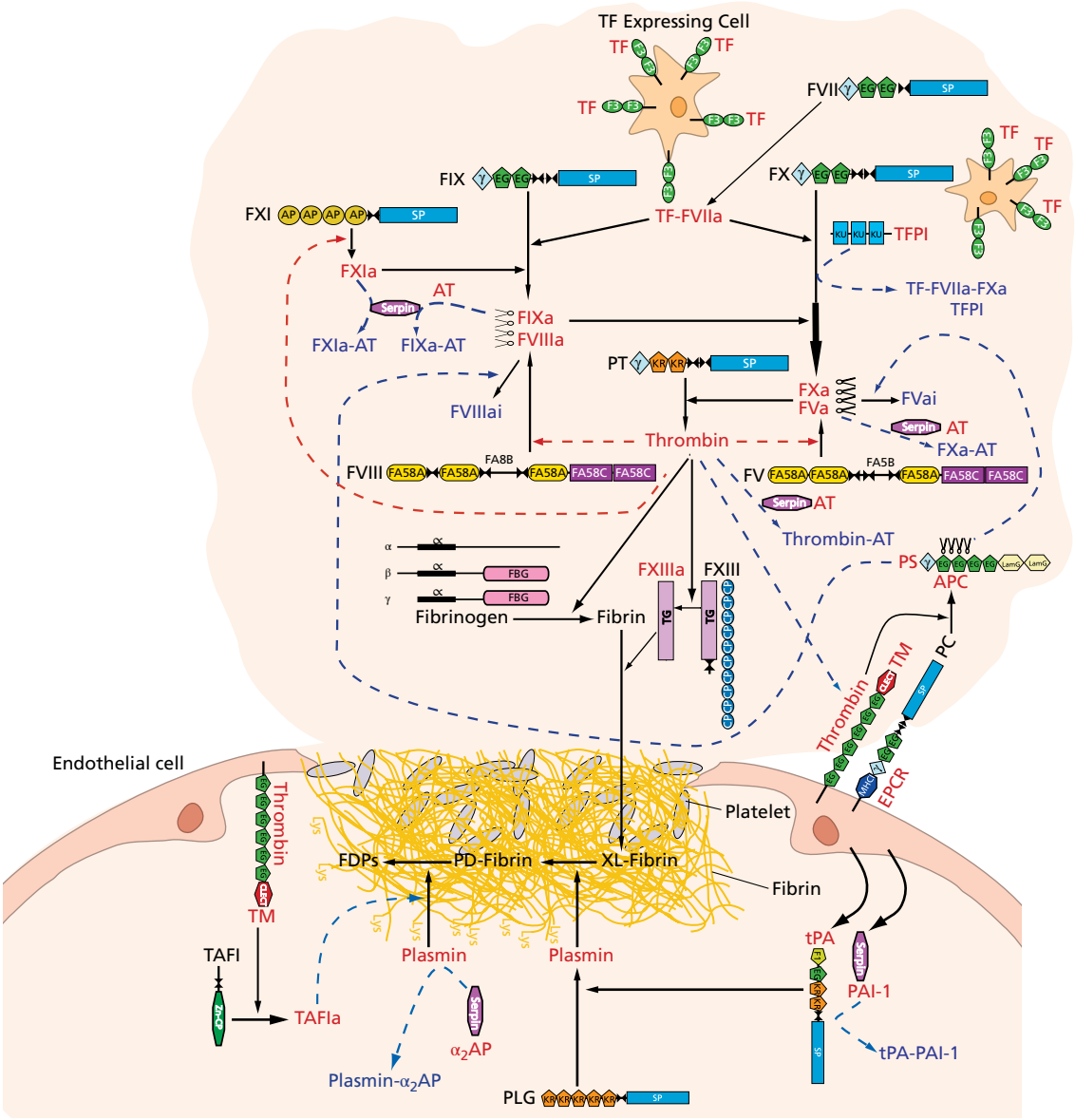

Figure 39.1 The haemostasis network. The network includes elements of coagulation initiation via TF–FVIIa interaction, amplification via the tenase (FVIIIa–FIXa) and prothrombinase (FVa–FXa) complexes, feedback inhibition through specific inhibitors and clot dissolution by fibrinolysis. Modular organisation of proteins is indicated (see Figure 39.8), and all abbreviations are given in the text. Protein names are represented as follows: functionally active proteins, red; inactivated or inhibited proteins, blue; precursors and fibrinogen-derived components, black. Lines and arrows are represented as follows: dashed red lines, positive procoagulant feedback loops; dashed blue lines, negative feedback and inhibitory loops; solid black lines, interactions or processes. Cream boxes indicate assembly of macromolecular complexes on a cellular phospholipid surface. The process of blood coagulation is initiated by the exposure of cells expressing TF to flowing blood. Thrombin generation is propagated by a series of positive feedback loops, leading to fibrin deposition. This process is controlled by a series of negative feedback steps: the initiation complex is inhibited by the formation of the quaternary complex TF–FVIIa–FXa–TFPI, and the active proteases FIXa, FXa and thrombin are inactivated by the serpin AT. In addition, thrombin initiates a negative feedback pathway by activating PC, leading to the inhibition of FVa and FVIIIa by specific proteolytic cleavages. The generation of fibrin leads to activation of the fibrinolytic pathway through binding of tPA, which leads to plasminogen activation and fibrin degradation. This pathway is also subject to inhibition through the binding of the serpins PAI-1 and α_2-antiplasmin (α_2AP) to tPA and plasmin, respectively. All these processes, plus platelet activation, occur simultaneously or with short lag times (Source: McVey and Tuddenham, 2007. Genomics of haemostasis. In: *Genomics and Clinical Medicine* (Kumar, ed.). Reproduced with permission of Oxford University Press.)

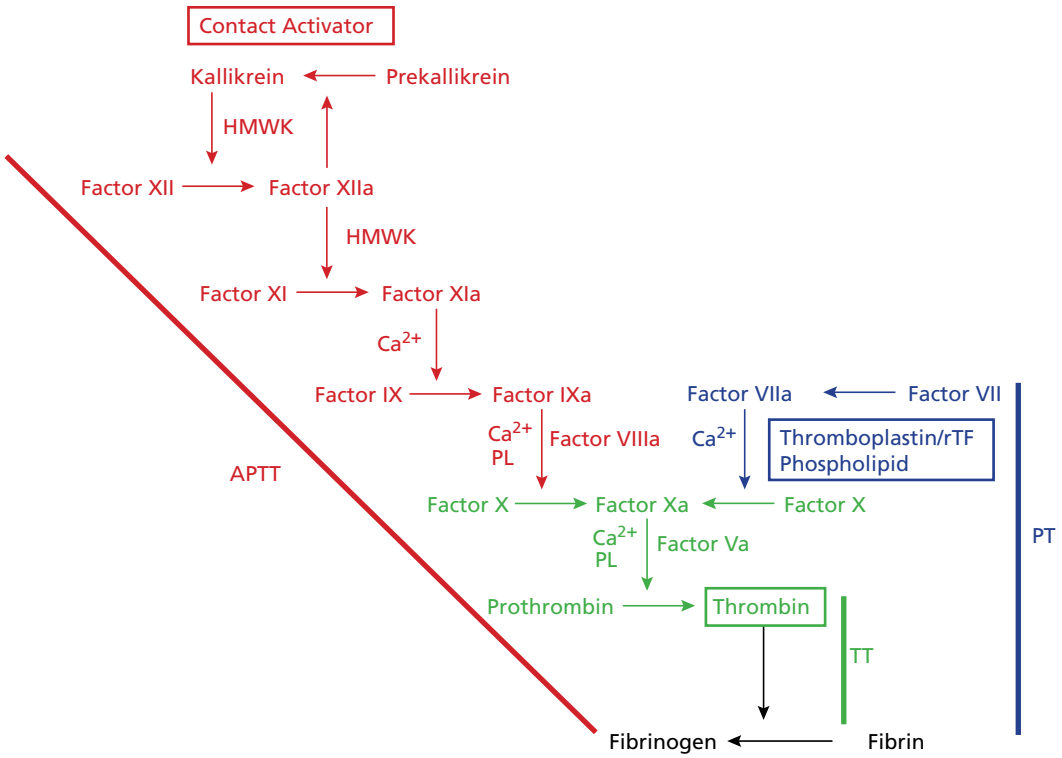

Figure 39.2 The coagulation cascade. The traditional concept of blood coagulation with separate intrinsic (red) and extrinsic (blue) pathways converging on the common pathway (green) with the generation of FXa. The activated partial thromboplastin time (APTT) is initiated by the addition of a contact activator (e.g. kaolin or silica) and calcium and tests for deficiencies in the intrinsic pathway. The prothrombin time (PT) is initiated by addition of thromboplastin (tissue factor), calcium and phospholipids and tests for deficiencies in the extrinsic pathway. The thrombin time (TT) is initiated by addition of thrombin and tests for an inhibitor of thrombin (most commonly heparin) or a problem with fibrin cleavage. HMWK, high-molecular-weight kininogen; PL, phospholipid.

varying the concentration of any component in such a system are not intuitively obvious.

Tissue factor initiates blood coagulation

Exposure of blood to cells expressing tissue factor (TF) on their surface is both necessary and sufficient to initiate blood coagulation *in vivo*, both in normal haemostasis and in pathological situations such as thrombosis. Figure 39.2 presents a version of the earlier cascade concept for intrinsic and extrinsic pathways of thrombin generation. Although the contact system does not appear to have a physiological role in haemostasis, it has emerged as a possible target for treatment of thrombosis without increasing the risk of bleeding. It also has to be considered during interpretation of the common coagulation tests that are used in clinical practice. TF is constitutively expressed at biological boundaries such as skin, organ surfaces, vascular adventitia and epithelial–mesenchymal surfaces, where it functions as a 'haemostatic envelope'. This ensures that, following disruption of vascular integrity, blood is immediately exposed to cells expressing TF, leading to the initiation of blood coagulation. The primary control of haemostasis is therefore the anatomical segregation of cells expressing functional TF from other components of the coagulation network present in blood.

Amplification of the initial stimulus

An updated concept of TF-initiated thrombin generation, including the important feedback reactions of thrombin, is shown in Figure 39.3. As stated above, it is now clear that the physiological initiator of blood coagulation is exposure of the circulating FVIIa and zymogen FVII to membrane-bound TF. Activation of FVII to the protease FVIIa and formation of a very high-affinity complex of TF with FVIIa result in the activation of FIX and FX by the TF–FVIIa (extrinsic tenase) complex. In the absence of its activated cofactor FVa, FXa generates only trace amounts of thrombin from prothrombin. Although insufficient to initiate significant fibrin polymerisation, thrombin formed in this initiation stage of coagulation is able to back-activate FV and FVIII by limited proteolysis. In the

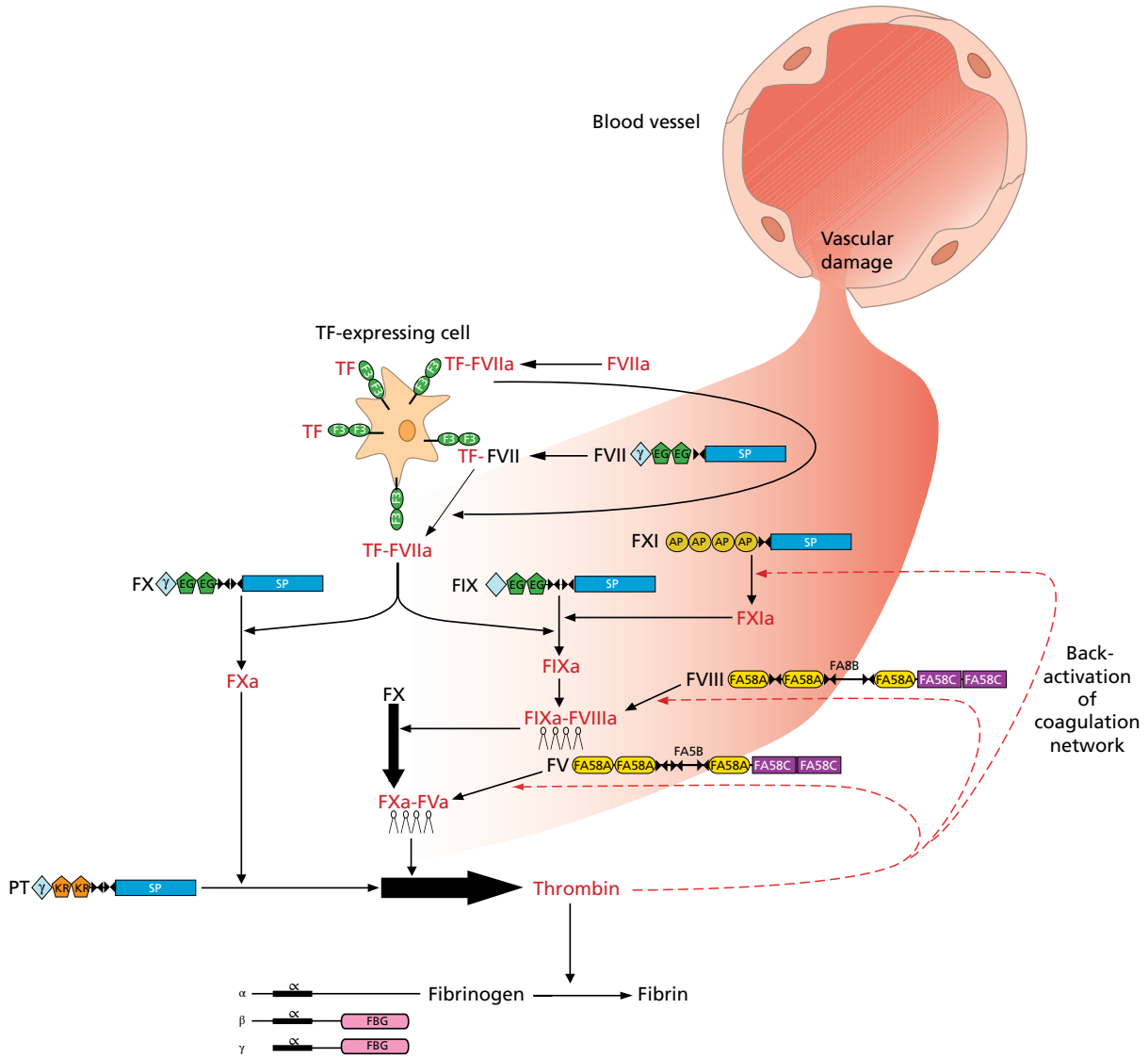

Figure 39.3 The haemostasis network: fibrin generation. Following vascular damage, blood coagulation is initiated by exposure of FVII/FVIIa to cells expressing the integral membrane protein TF. Activation of FVII to the protease FVIIa results in the activation of FIX and FX by the TF–FVIIa complex. In the absence of its activated cofactor FVa, FXa generates only trace amounts of thrombin. Although insufficient to initiate significant fibrin polymerisation, trace amounts of thrombin formed in this initiation stage of coagulation are able to back-activate FV and FVIII by limited proteolysis. In the amplification phase of coagulation, FVIIIa forms a complex with FIXa, activating sufficient FXa, that (in complex with FVa) leads to the explosive generation of thrombin, ultimately leading to generation of a fibrin clot. The tenase (FVIIIa–FIXa) and prothrombinase (FVa–FXa) complexes assemble on phospholipid surfaces. Colours and symbols as in Figure 39.1.

amplification phase of coagulation, FIXa forms a complex with FVIIIa. This is the intrinsic tenase complex (FVIIIa–FIXa) that activates sufficient FX to FXa to form a complex with FVa, producing the prothrombinase complex (FVa–FXa). This results in the explosive generation of thrombin, that ultimately leads to generation of a fibrin clot. Thrombin can also back-activate FXI to FXIa by limited proteolysis, and FXI further activates FIX to FIXa, providing further amplification.

A key feature of these processes is the assembly of multimolecular complexes on a phospholipid surface provided *in vivo* by cell membranes. For procoagulant complexes such as tenase and prothrombinase, this surface is provided by activated platelets. Each of these complexes consists of a cofactor (TF, FVa and FVIIIa), an enzyme (FVIIa, FIXa and FXa) and a substrate that is a zymogen (FIX, FX and prothrombin) of a serine protease. The product of one reaction becomes the

enzyme in the next complex. The procofactors FV and FVIII are themselves activated by limited proteolytic digestion.

Feedback inhibition of the procoagulant response

The TF–FVIIa complex is rapidly inactivated by tissue factor pathway inhibitor (TFPI), released from thrombin-activated platelets or found on the surface of endothelial cells, by the formation of a quaternary inhibited complex (Figure 39.4). This explains the requirement for further activation of FX via FIXa–FVIIIa, and back-activation of FXI by thrombin, to permit further activation of FIX. Figure 39.4 also indicates the role of one of the other important inhibitors of the network, antithrombin (AT), which damps down thrombin generation by forming inactive complexes with FIXa, FXa, FXIa and

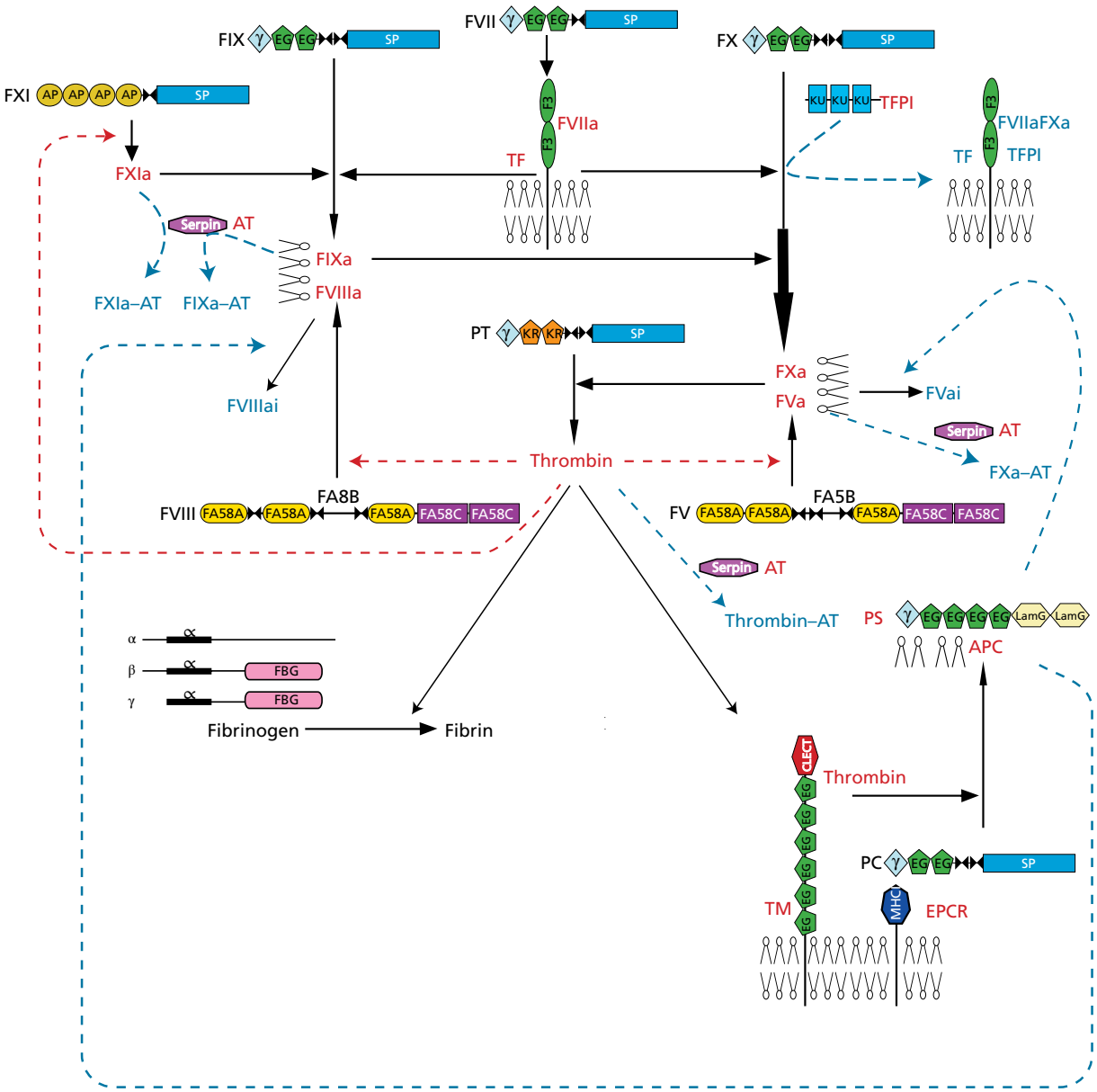

Figure 39.4 The haemostasis network: inhibition of thrombin generation. The initiation of blood coagulation via generation of FIXa and FXa by TF–FVIIa is shut down by the action of TFPI, which forms a quaternary complex with TF–FVIIa–FXa. Further generation of FIXa occurs following back-activation of FXI by thrombin. The serine proteases FIXa, FXa, FXIa and thrombin are all inhibited by AT. At the endothelial cell surface, thrombin bound to TM activates PC bound to its receptor EPCR. APC in complex with its cofactor PS inactivates FVa and FVIIIa by further proteolytic cleavages. Colours and symbols as in Figure 39.1.

thrombin. The rate of inhibition by AT is substantially increased by binding a variety of polysulfated mucopolysaccharides known as glycosaminoglycans (GAGs) on the surface of endothelial cells.

Thrombin also proteolytically activates an anticoagulant pathway, the protein C (PC) pathway (Figure 39.4). Thrombomodulin (TM), an endothelial cell surface receptor, plays a pivotal role in the activation of this anticoagulant pathway. TM binds thrombin and, by an allosteric mechanism, alters its substrate specificity. The procoagulant substrates of thrombin, including FV, FVIII and fibrinogen, are no longer efficiently proteolysed. The preferred substrate of the thrombin–TM complex is PC, a zymogen that is proteolysed to activated PC (APC). Thrombin binding to TM also enhances the substrate specificity to thrombin-activatable fibrinolysis inhibitor (TAFI). The activation of PC can be augmented by another cellular receptor, endothelial cell protein C receptor (EPCR), which provides a direct endothelial cell binding site for PC and thus increases the apparent affinity of the thrombin–TM complex for PC; however, not all endothelial cells coexpress TM and EPCR. In complex with its cofactor, protein S (PS), APC rapidly inactivates the procoagulant cofactors FVa and FVIIIa by specific proteolysis, forming a negative feedback loop. The PC pathway is most active in the microvasculature, where the relative density of TM is highest. *EPCR* expression by contrast is largely restricted to large vessels.

Fibrinolysis

The final step in the regulation of fibrin deposition is the prevention and/or rapid removal of insoluble fibrin by the fibrinolytic system (Figure 39.5) described more fully later in the chapter. Briefly, once sufficient fibrin is generated, it binds

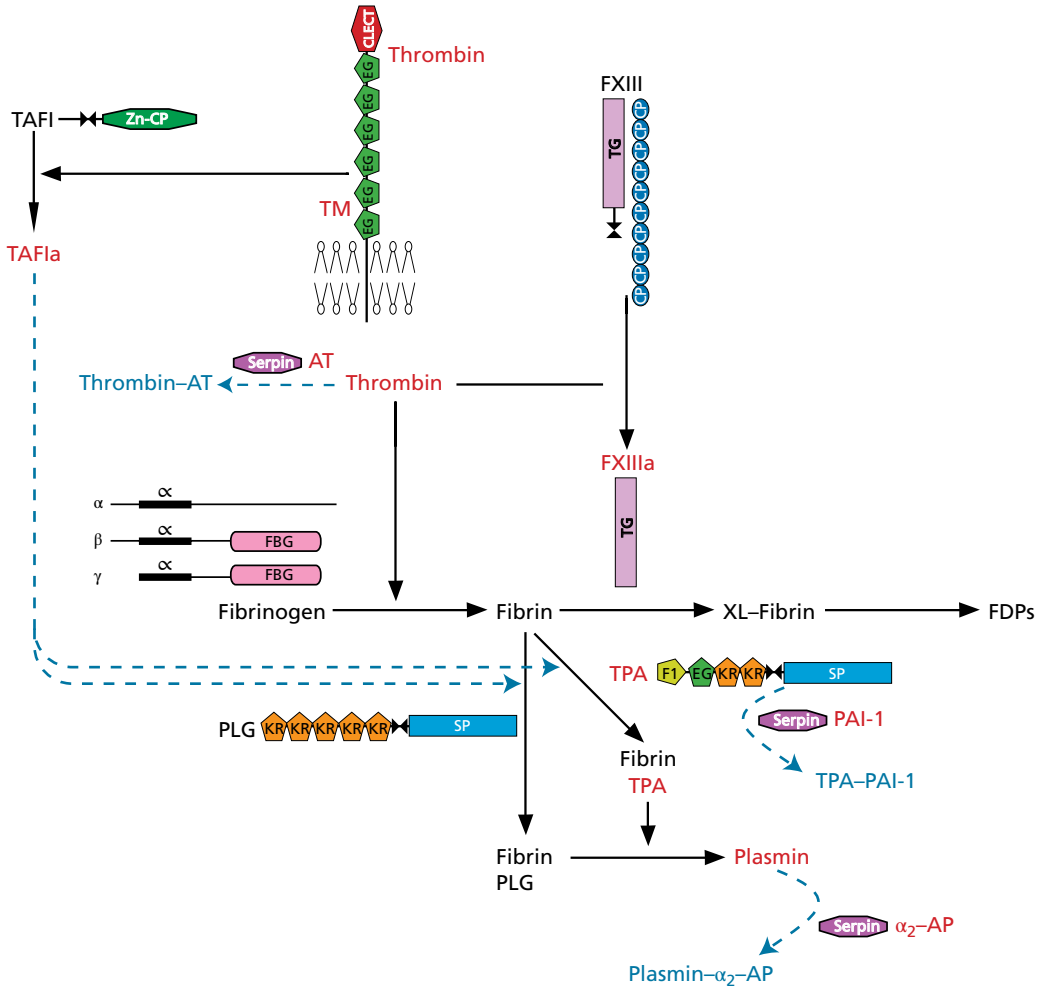

Figure 39.5 The haemostasis network: fibrinolysis. Both PLG and tPA bind to polymerised fibrin, promoting the cleavage of PLG by tPA to active plasmin, which then degrades cross-linked fibrin to soluble FDPs. Modulation of fibrinolysis is achieved by serpin inhibition: PAI-1 inactivates tPA, while α_2-antiplasmin (α_2AP) inhibits plasmin. Conversely, TAFIa removes Lys residues from fibrin, removing PLG- and tPA-binding sites, reducing plasmin generation. Colours and symbols as in Figure 39.1.

tissue plasminogen activator (tPA), leading to the increased activation of plasminogen (PLG). This results in the formation of plasmin at the site of the fibrin clot, which breaks down fibrin into soluble fibrin degradation products. The fibrinolytic system is also subject to inhibition through the action of inhibitors of tPA and plasmin, namely, plasminogen activator inhibitor (PAI)-1 and α_2-antiplasmin, respectively. In addition, the activated TAFI generated by the action of TM-thrombin serves as a negative regulator of fibrinolysis, enhancing the stability of fibrin clots.

Blood vessels

The endothelium

The endothelium functions in a multitude of physiological processes, including intracellular transport, the regulation of vasomotor tone and maintenance of blood flow. Endothelial cells possess surface receptors for a variety of physiological substances, for example thrombin and angiotensin II, which may influence vascular tone directly or indirectly through various haemostasis-related events.

The apical luminal surface of the endothelium has a polysaccharide-rich layer mainly composed of glycosaminoglycans, proteoglycans and glycoproteins known as the glycocalyx. It creates a barrier between circulating cells/molecules and the vessel wall. It plays a key role in regulating a number of processes: it participates in angiogenesis, exerts an anticoagulant effect, prevents leukocyte adhesion, acts as a selective permeable barrier and filter, operates as a mechano-transducer of shear stress and contributes to maintaining vascular tone. Heparan sulfate proteoglycan is the predominant constituent and bears a significant negative charge at physiological pH, which promotes electrostatic interactions with positively charged residues on circulating proteins such as AT, HCII and TFPI exerting an anticoagulant effect. Endothelial activation is characterised by shedding of the glycocalyx and increased expression of adhesion molecules.

Once activated, endothelial cells express a variety of intracellular adhesion molecules, some of which are released into the plasma. These include vascular cell adhesion molecule (VCAM), E-selectin, P-selectin, and von Willebrand factor (VWF), which modulate leucocyte and platelet adhesion, inflammation, phagocytosis and vascular permeability.

However, the endothelium should not be regarded as a simple homogeneous cell type. It would appear that endothelial cell phenotypes are differentially regulated. At any given point in time, structural and functional phenotypes may vary between segments of the vascular tree, and at any given location, the endothelial phenotypes may change from one moment to the next. Endothelial cell heterogeneity occurs between different organs, within the vasculature of a given organ, and even between neighbouring endothelial cells of a single vessel.

The platelet–vessel wall interaction

Healthy unperturbed endothelial cells exert a powerful inhibitory influence on haemostasis by virtue of the factors they release or express on their surface (see Figure 39.6a). Two of these, prostaglandin (PG)I_2 (or prostacyclin) and nitric oxide (NO), also known as endothelium-derived relaxing factors, have powerful vasodilator activity, acting on smooth muscle cells in the vessel wall (basal-directed secretion) and hence modulating blood flow. Both substances inhibit aggregation of platelets and leucocytes (luminal-directed secretion) by increasing intraplatelet levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), respectively (see below).

PGI_2 is the major prostaglandin synthesised by endothelial cells, a small amount also being produced by fibroblasts and smooth muscle cells. Its action on platelets involves binding to a specific G-protein-coupled receptor (PTGIR) that activates adenylate cyclase, thus increasing the intraplatelet cAMP concentration. This promotes Ca^{2+} uptake into the dense tubular system and inhibits phosphatidylinositol metabolism, both of which prevent platelet aggregation and the consequent release of storage granules containing procoagulant molecules such as VWF and FV. Thrombin, and other agents that are generated at the site of injury, stimulate the synthesis of PGI_2 by adjacent endothelial cells, which counteracts the platelet-aggregating activity of the protease and thereby helps to localise platelet plug formation. PGI_2 is rather unstable, with a half-life of only a few minutes in whole blood, and degrades spontaneously to 6-keto-$PGF_{1\alpha}$. This degradation product is biologically inert, but in some tissues, a variable proportion of PGI_2 may be enzymatically converted to 6-keto-$PGF_{1\alpha}$ or 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$, which retains some platelet-inhibitory activity.

The once-held belief that PGI_2 is a circulating hormone that limits platelet–platelet interactions within the bloodstream is probably false, as plasma levels of PGI_2 are at least two orders of magnitude below than that needed to inhibit platelet aggregation. Although in some settings, such as following severe trauma, it is possible that markedly increased systemic levels could occur transiently, it is more likely that PGI_2 serves mainly as a local hormone, principally concerned with vascular tone, but possibly also inhibiting the extension of the platelet plug beyond the immediate vicinity of any endothelial damage.

NO is synthesised in smooth muscle cells, macrophages and activated platelets, as well as by endothelial cells. Its synthesis and secretion may be constitutive (when it serves as a local hormone to 'fine tune' blood flow). Stimulated (inducible) synthesis of NO also occurs in endothelial cells exposed to cytokines such as interleukin (IL)-1, tumour necrosis factor (TNF) or endotoxin. This produces a slower but longer-lasting increase in NO level and can have undesirable side-effects, such as inflammation, cytotoxicity or prolonged hypotension. Like PGI_2, it has a very short half-life (3–5 s) and is rapidly oxidised to the inactive nitrite (NO_2^-) or nitrate (NO_3^-) forms.

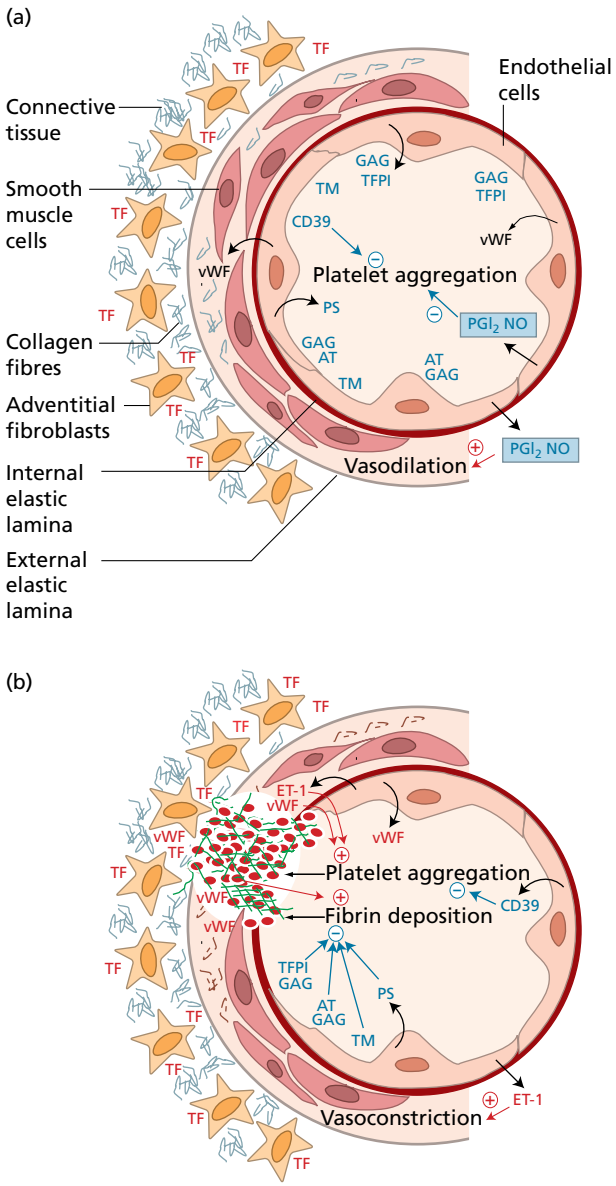

Figure 39.6 Blood vessel architecture and functions in haemostasis. (a) The anatomy of a generic vessel in the resting state. The layer thicknesses vary widely between arterial and venous circulation and between large and small vessels. In the absence of vessel damage, cells expressing TF are anatomically separated from components of the blood coagulation network, preventing initiation of coagulation. In addition, coagulation inhibitors present on the endothelial surface and inhibitors of platelet aggregation maintain vessel patency. (b) The localised haemostatic response to vessel injury: initiation of coagulation by TF exposure and platelet activation/aggregation by exposure of extravascular collagen to plasma VWF. Procoagulant responses away from the lesion site are damped down by anticoagulant and antiplatelet factors derived from the endothelium.

In addition to PGI_2 and NO, endothelial cells also express an ecto-ADPase (CD39) on their cell surface, which rapidly metabolises ATP and ADP, released from activated platelets, to AMP, thereby drastically reducing or abolishing platelet recruitment and aggregation in response to thrombin.

The 21-amino-acid peptide endothelin (ET)-1 is the predominant isoform of the endothelin peptide family, which also includes ET-2, ET-3 and ET-4. It exerts various biological effects, including vasoconstriction and the stimulation of cell proliferation in tissues, both within and outside the cardiovascular system. In the endothelium, ET-1 is predominantly released abluminally towards the vascular smooth muscle, where it acts as a potent vasoconstrictor and mitogen for smooth muscle cells. In addition, ET-1 stimulates the production of cytokines and growth factors. Furthermore, it stimulates neutrophil adhesion and platelet aggregation and is chemotactic for macrophages.

Thrombospondin (TSP)-1 is an abundant constituent of the α-granules of platelets and Weibel–Palade bodies of endothelial cells. TSP-1 expression is upregulated by vascular cells after injury and in chronic disease. Upon release, TSP-1 binds to the cell surface in a Ca^{2+}-dependent manner and interacts with a number of cellular membrane proteins, including integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, CD36 and the integrin-associated protein (IAP or CD47) and integrin-bound fibrinogen and fibronectin. TSP-1 appears to function by regulating the formation of multiprotein complexes that modulate cellular phenotype. The cellular response varies depending on the cell type as each cell expresses

a different repertoire of receptors. On the platelet, TSP-1 activates $\alpha_{IIb}\beta_3$ through its binding to IAP. This results in platelet spreading and aggregation.

von Willebrand factor

VWF is a multimeric glycoprotein that plays an important role in primary haemostasis by promoting platelet adhesion to the subendothelium at sites of vascular injury under high shear rate conditions. It is also a carrier of FVIII, and this association protects FVIII from rapid proteolysis and clearance. Failure of FVIII to bind VWF, as occurring in type 2N von Willebrand disease (see Chapter 41), leads to its rapid turnover and resulting FVIII deficiency. VWF is synthesised by endothelial cells, megakaryocytes and platelets. In endothelial cells, VWF may be secreted directly into the circulation or stored in Weibel–Palade bodies. The VWF produced in megakaryocytes and platelets is not secreted but stored in α-granules. Release of VWF from intracellular stores occurs following activation of endothelial cells or platelets. Some therapeutic products, such as desmopressin, work by stimulating release of stored VWF.

Mature VWF is composed of disulfide-linked subunits, each comprising 2050 amino acid residues and up to 22 carbohydrate side-chains. Each subunit has a molecular mass of approximately 278 kDa, of which 10–19% is carbohydrate. Two subunits joined at their C-termini by disulfide bridges form a dimer. Additional disulfide bridges between dimers lead to the formation of larger multimers that range in size from 500 kDa to in excess of 10,000 kDa (Figure 39.7). The ultra-large VWF multimers are predominantly stored, but when secreted by endothelial cells may appear as globular molecules or as thin filaments that are several microns long. These ultra-large VWF multimers can only be detected transiently in normal plasma because they are cleaved by a plasma metalloproteinase (ADAMTS-13). Deficiency or inhibition of ADAMTS-13 results in accumulation of ultra-large VWF multimers in plasma, leading to enhanced shear-induced platelet aggregation and microvascular thrombosis, as seen in thrombotic thrombocytopenic purpura (TTP) (see Chapter 46).

VWF contains binding sites for collagen, fibrinogen and the platelet receptor, glycoprotein (GP)Ib. When vascular injury exposes subendothelial collagen, VWF binds and becomes unwound from its globular form (Figure 39.7c). This exposes more binding sites and allows the capture of platelets from the circulation. Thus VWF and, to some extent, fibrinogen act as bridges between the platelet and the injured vascular wall. Variants in the genes encoding the GPIbα complex that lead to loss of function cause the bleeding disorder Bernard–Soulier syndrome. Conversely, gain-of-function variants in either VWF or GPIb that increase the interaction lead to type 2B von Willebrand disease or pseudo (platelet-type) von Willebrand disease (see Chapter 41).

Endothelial cell anticoagulant activities

Endothelial cells regulate coagulation by a number of mechanisms. The activity of the PC pathway is promoted by expression of two cellular receptors. TM is constitutively expressed on all endothelial cells, with the exception of the brain. EPCR is expressed strongly in the endothelial cells of arteries and veins in the heart and lung, less intensely in capillaries in the lung and skin, and not at all in the endothelium of small vessels of the liver and kidney. In addition, endothelial cells synthesise and secrete PS, the cofactor for APC in the inactivation of FVa and FVIIIa.

Endothelial cells regulate the initiation of coagulation by synthesising the main physiological inhibitor of the FVIIa–TF initiating complex, namely, TFPI. This is described in greater detail below. Endothelial cell-surface GAGs are important in binding TFPI, AT and HCII and also modulate the activity of AT, which inhibits thrombin and FXa, and to a lesser extent FIXa and FXIa.

Endothelial cell-derived fibrinolytic factors

Three important fibrinolytic factors are detectable in the vessel wall: tPA and PAI-1 are synthesised primarily by endothelial cells, whereas urinary plasminogen activator (uPA, urokinase) is mainly derived from fibroblast-like cells in the kidney and gut. Unlike VWF and P-selectin, tPA and PAI-1 are not stored in Weibel–Palade bodies and, in the resting state, synthesis and secretion are slow, resulting in low circulating levels. However, stimulated synthesis and release occur in response to a variety of stimuli.

Coagulation factors

A summary of the characteristics of the proteins discussed, including gene location, number of exons, molecular mass, circulating concentration, plasma half-life and presumed function, can be found in Table 39.1. The domain structure and modular nature of the proteins are shown in Figure 39.8. This demonstrates the similarity between coagulation factors that belong to the same protein family, such as the serine proteases (e.g. compare FVII, FIX, FX and PC). Many of the coagulation proteins were traditionally numbered from the mature N-terminus of the protein as +1, with the signal peptide and propeptide numbered negatively. However, following attempts to standardise systems for naming genes and variations within them, it was recommended that proteins should be numbered from the initiation methionine as +1 according to guidelines from the Human Genome Variation Society (HGVS). To reduce confusion, all amino acids in this chapter adhere to this guidance, but the reader should be aware that many historical publications will use this legacy numbering system, and therefore legacy descriptions are included in parentheses for clarity.

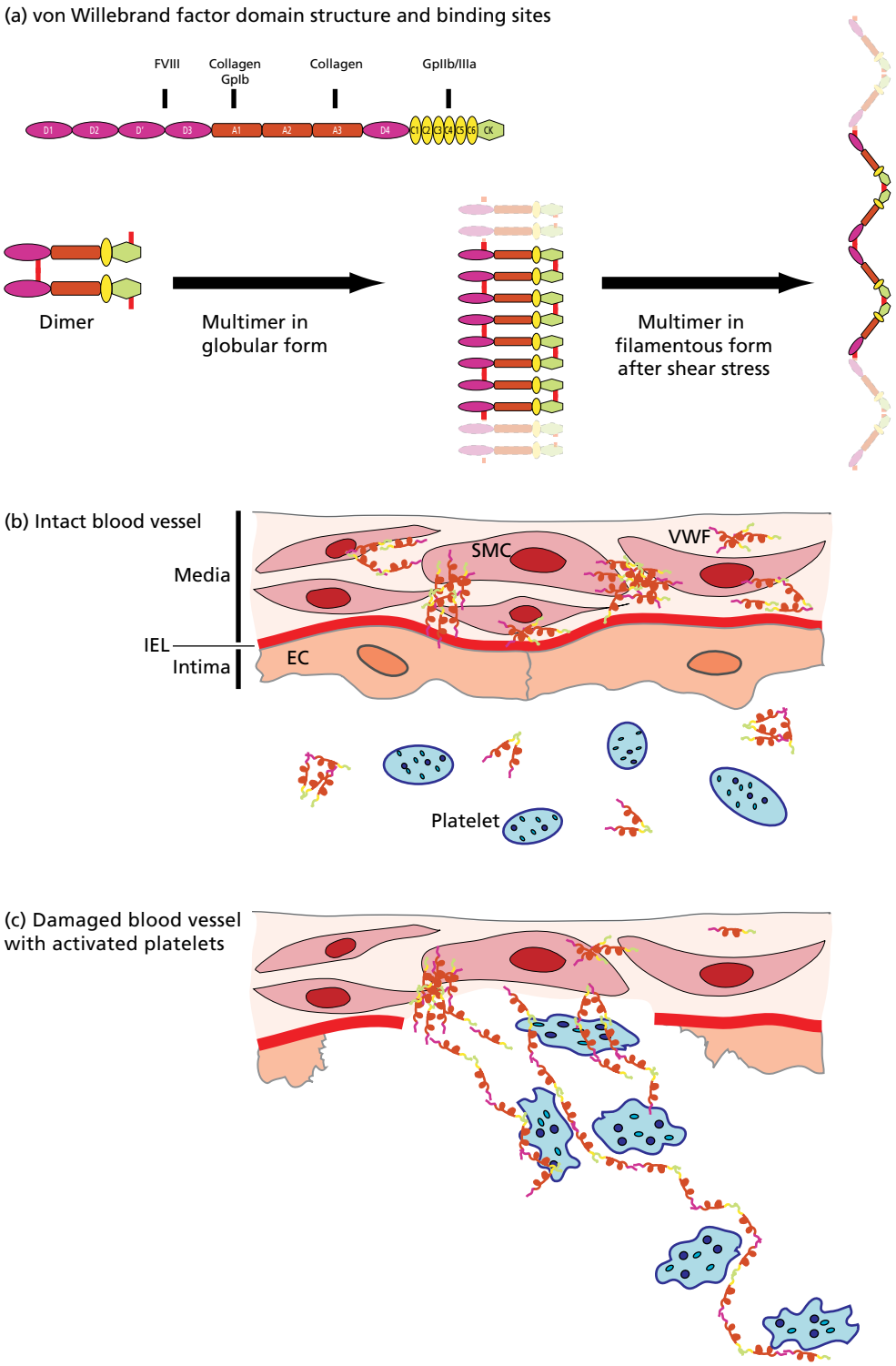

Figure 39.7 Role of von Willebrand factor (VWF) in haemostasis. (a) VWF domain structure of the primary translation product. The 22-amino acid secretory peptide (SP) is cleaved off after translocation of the primary translation product to the endoplasmic reticulum. Dimers are formed when individual monomers become joined through disulfide bonds at the C-terminus. Multiple dimers are then joined at the N-terminus to form high-molecular-weight multimers that take on a globular shape. This latter process requires the propeptide (D1–D2) which is then cleaved off in the trans-Golgi. When exposed to flowing blood under high shear stress, the multimers become filamentous, exposing their binding sites. (b) In the normal vasculature, the ultra-high-molecular-weight VWF multimers required for full VWF function are found only in the subendothelium. The circulation contains lower-molecular-weight multimers. After disruption of the endothelium (c), exposure of high-molecular-weight multimers to flowing blood applies shear stress, causing the multimers to unravel and capture activated platelets. The coagulation network forms on the surface of these platelets. Thus, VWF acts to localise the process of clot formation to the site of vascular damage. EC, endothelial cell; IEL, internal elastic lamina; SMC, smooth-muscle cell.

Table 39.1 Key proteins involved in the haemostatic network.

Common name	Abbreviation	Gene symbol	Gene location	No. of exons	Amino acids (mature)	M_r of monomer (kDa)	Plasma level (μg/mL)	Plasma level (nmol/L)	$t_{½}$ (hours)
Tissue factor	TF	*F3*	1p22–p21	6	263	44	NA	NA	NA
Prothrombin	FII	*F2*	11p11.1	14	579	72	90	1400	65
Factor V	FV	*F5*	1q23	25	2196	330	10	30	15
Factor VII	FVII	*F7*	13q34	8	416	50	0.5	10	3
Factor VIII	FVIII	*F8*	Xq28	26	2332	330	0.1	0.3	10
Factor IX	FIX	*F9*	Xq27	8	415	56	5	90	25
Factor X	FX	*F10*	13q34	8	445	59	8	135	40
Factor XI	FXI	*F11*	4q35	15	607	80*	5	30	45
Prekallikrein	PK	*KLKB1*	4q35	15	638	86	50	580	35
Factor XIII† (A chain)	FXIII	*F13A1*	6p25.3–p24.3	15	731	75†	10	30	200
Factor XIII† (B chain)	FXIII	*F13B*	1q31	12	641	80†	10	30	200
Fibrinogen (α-chain)‡	FGN	*FGA*	4q28	6	866	68‡	3000	9000	90
Fibrinogen (β-chain)‡	FGN	*FGB*	4q28	8	491	52‡	3000	9000	90
Fibrinogen (γ-chain)‡	FGN	*FGG*	4q28	10	453	49‡	3000	9000	90
von Willebrand factor	VWF	*VWF*	12p13.3	52	2050	255	10	40	12
Thrombomodulin	TM	*THBD*	20p11.2	1	557	60	NA	NA	NA
Endothelial protein C receptor	EPCR	*PROCR*	20q11.2	7	220	27	NA	NA	NA
Protein C	PC	*PROC*	2q13–14	9	419	62	4	65	6
Protein S	PS	*PROS1*	3q11.2	15	676	69	10 (free)	145	?
Tissue factor pathway inhibitor	TFPI	*TFPI*	2q32	12	304	42	0.08	2.5	?
Antithrombin	AT	*SERPINC1*	1q23–q25.1	9	464	58	140	2400	45
Heparin cofactor II	HCII	*SERPIND1*	22q11.21	5	499	66	90	1200	60
Plasminogen	PLG	*PLG*	6q26	14	791	92	200	2000	50
Tissue plasminogen activator	tPA	*PLAT*	8p12	14	562	69	0.005	0.07	0.03
Prourokinase	UK	*PLAU*	10q24	11	431	54	0.0015	0.04	0.03
Plasminogen activator inhibitor 1	PAI-1	*SERPINE1*	7q21.3–q22	9	379	52	10	200	0.1
α_2 -Antiplasmin	α_2-AP	*SERPINF2*	17p13	9	452	67	70	1000	72
Thrombin-activatable fibrinolysis inhibitor	TAFI	*CPB2*	13q14.11	11	401	60	5	75	0.2

* FXI circulates as a 160-kDa homodimer of two 80-kDa monomers.
† FXIII circulates as a 326-kDa tetramer of two A- and two B-chains.
‡ Fibrinogen circulates as a 340-kDa complex of two each of A-, B- and C-chains.

Tissue factor

The formation of the FVIIa–TF complex is regarded as the sole initiator of coagulation in both normal and pathological coagulation. The processed mature TF protein is a 263-amino acid protein after signal peptide cleavage. The 219-residue extracellular region consists of two fibronectin type III domains anchored into the cell membrane by a 23-residue transmembrane sequence. The extracellular region binds tightly to FVIIa to form a highly active procoagulant complex. The 21-residue cytoplasmic domain has been implicated in intracellular signalling; however, transgenic experiments in mice have shown that this domain is not required for either development or procoagulant function, so its role is still unclear.

Vascular adventitial cells, neuroglia, vascular smooth muscle and epidermal cells express TF constitutively. It therefore forms a protective envelope around blood vessels and organs, ready to initiate clotting as soon as blood leaks out of vessels. TF is also expressed by monocytes and endothelium after activation by inflammatory cytokines or by endotoxin, as occurs in sepsis, and

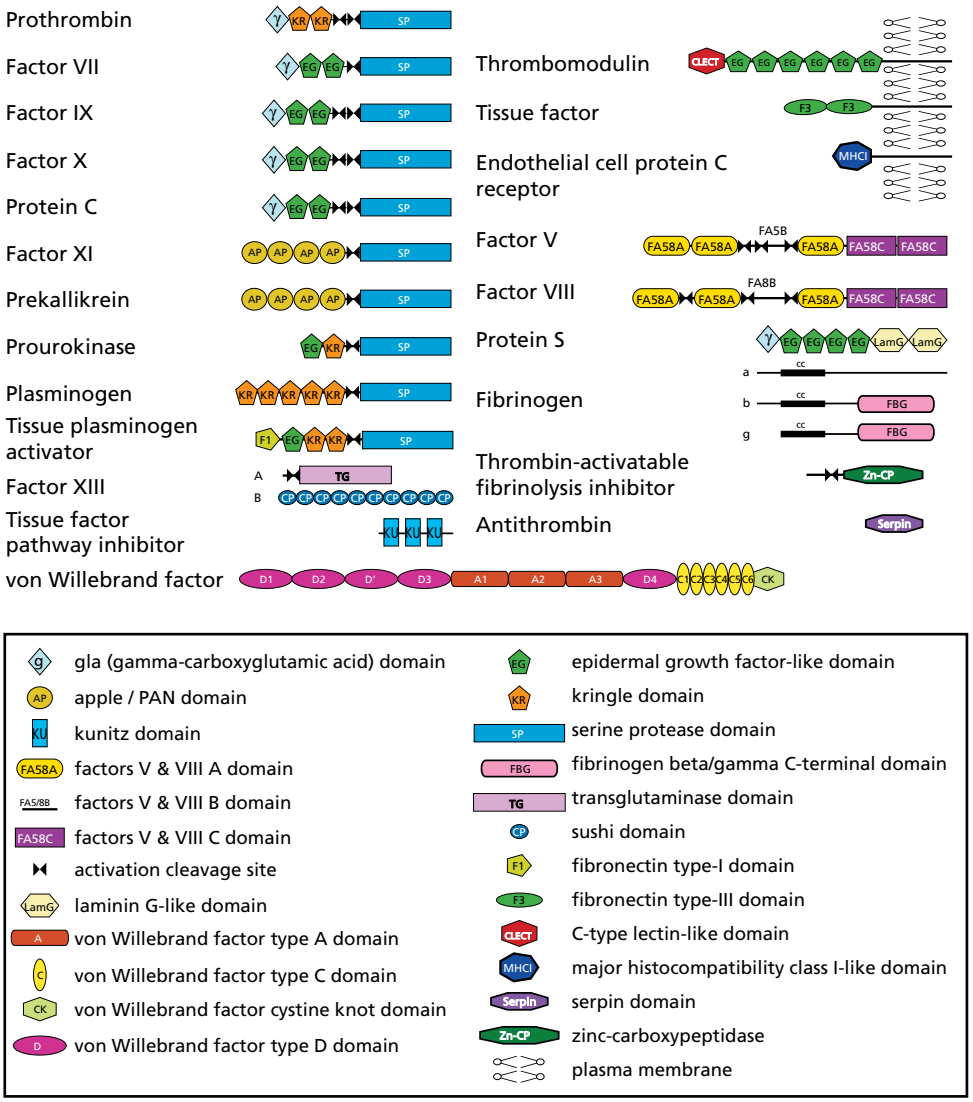

Figure 39.8 Modular organisation of haemostasis proteins. The evolutionary relationship of many factors is suggested by their modular organisation.

on cancerous tissues. Intravascular exposure of TF by any route, but particularly after atherosclerotic plaque rupture, can result in pathological thrombosis. Recent work has also focused on the novel hypothesis that functionally active TF can circulate in flowing blood on small procoagulant extracellular vesicles (exosomes), which may also be transferred from one blood cell type to another via specific receptor-mediated interactions. These procoagulant extracellular vesicles are often derived from tumour cells and can promote metastasis and pre-metastatic niche formation.

Factor VII

Plasma FVII/FVIIa binds to TF, for example after vessel trauma or plaque rupture, to form a complex that initiates coagulation by directly activating FX and to a lesser extent FIX. Approximately 1–2% of total circulating *FVII* is in the active form FVIIa. The FVII gene (*F7*) lies adjacent to the factor X gene (*F10*), suggesting gene duplication during evolution, and close to that of protein Z (*PZ*) at 13q34. In common with the other serine proteases of the coagulation network (FIX, FX, prothrombin and PC), as well as PS and PZ, FVII has an N-terminal domain that contains a number (9–12) of glutamic acid residues that are post-translationally modified by the addition of a carboxyl group to the γ-carbon by a vitamin K-dependent carboxylase. This γ-carboxyglutamic acid (GLA) domain confers affinity to negatively charged phospholipid membranes, such as those of activated platelets or endothelial cells, promoting the assembly of functional multiprotein complexes on these surfaces. Proteins containing GLA modules are commonly referred to as 'vitamin

K-dependent factors' as this vitamin is a cofactor in the carboxylation reaction. Blocking this post-translational modification with coumarin derivatives such as warfarin was one of the main treatments for the long-term prevention of thromboembolic events prior to the development of direct oral anticoagulants (DOACs). FVII in common with other GLA domain-containing proteins has a primary translation product that contains a propeptide sequence that directs the γ-carboxylation at 10 Glu residues within the GLA domain. The GLA domain is followed by two epidermal growth factor (EGF)-like domains, the connecting or activation peptide and the serine protease domain. FVII is activated by cleavage between residues Arg212 (152) and Ile213 (153), yielding a two-chain disulfide-linked FVIIa molecule; however, this has little catalytic activity until it is bound to TF. The half-life of FVII zymogen in plasma is 3–6 hours and, remarkably, the half-life of the FVIIa enzyme is 2–3 hours, probably because there is no plasma inhibitor capable of effectively neutralising free FVIIa. Recombinant FVIIa is in clinical use as a treatment for haemophilia with inhibitors and other severe heritable or acquired bleeding disorders.

Factor X

The gene (*F10*) and protein structures of FX closely resemble those of FVII. Unlike FVII, zymogen FX circulates as a two-chain disulfide-linked heterodimer due to excision of a tribasic peptide (Arg-Lys-Arg) at residues 179–181 (139–141) during synthesis. The light chain of FX therefore consists of the GLA domain with 11 γ-carboxyglutamic acid residues, the amphipathic helix and two EGF-like domains. The half-life of FX in plasma is 36 hours. FX is activated by either FIXa–FVIIIa or TF–FVIIa on phospholipid surfaces in the presence of Ca^{2+} ions by proteolytic cleavage at Arg234-Ile235 (Arg194-Ile195). FXa forms a phospholipid-bound complex with FVa, which efficiently activates prothrombin (prothrombinase complex).

Factor IX

The FIX gene (*F9*) is located at Xq26, about 15.2 Mb from the FVIII gene (*F8*); thus, deficiencies of both factors are X-linked disorders. Deficiency of FIX results in clinical haemophilia B, as the main function of FIX is to participate in the tenase complex (FIXa–FVIIIa). The FIX promoter has been extensively studied and includes sites for liver-specific transcription factors. The 5′ untranslated region of the *F9* gene has also been extensively studied and includes two sites for age-related gene regulation that play a role in the haemophilia B Leyden phenotype (FIX deficiency, which corrects spontaneously at puberty). The plasma half-life of FIX is 18 hours.

The mature protein is a single-chain molecule consisting of 415 amino acids, and the GLA domain contains 12 γ-carboxyglutamic acid residues. Either TF–FVIIa or FXIa cleaves FIX within the connecting peptide (between residues 191 and 192 (145–146) and 226 and 227 (180–181)). Unlike FVII and FX, FIX requires two proteolytic cleavages, releasing an activation peptide, in order to achieve full activation.

Factor XI

FXI is a zymogen of a serine protease that has four Apple (PAN1–4) domains and a serine protease domain in each monomer. The protein circulates as a homodimer, with the two monomer subunits linked via a series of interactions between the two Apple four domains that includes a disulfide bridge. Activation of FXI is by a single cleavage performed by thrombin. FXIa then activates FIX directly in free solution. FXI activation by the contact protein FXIIa was originally thought to be a relevant step in intrinsic or contact-activated coagulation (see Figure 39.2), but it is now considered that the feedback activation of FXI by trace thrombin provides a physiologically relevant route for generation of increased amounts of FIXa to assemble tenase during the amplification of the initial TF stimulus (see Figure 39.3). Thrombin activates FXI by proteolytic cleavage at Arg387-Ile388 (Arg369-Ile370).

There is increasing evidence that FXI has other roles in haemostasis, such as regulation of fibrinolysis by the thrombin-activatable fibrinolysis inhibitor (TAFI). The fact that bleeding symptoms in FXI deficiency correlate poorly with FXI procoagulant level, suggesting that these other functions may be important in determining the phenotype in this mild bleeding disorder.

Factor XIII

FXIII circulates as a tetramer of two A-chains and two B-chains. The B-chains function as carriers for the A-chains which, after activation by thrombin, function as a transglutaminase to cross-link fibrin and other proteins in the clot, notably antiplasmin, resulting in a stable structure. FXIIIa contains a free sulfydryl group at the active site. Platelets also contain FXIII A-chain dimers, which are fully functional after thrombin activation.

Factor VIII

The FVIII gene (*F8*) spans 187 kb of the X chromosome (Xq28) and encodes a protein of 2351 amino acids. After removal of the 19-amino acid signal sequence, a single chain of 2332 amino acids is transiently formed but is subsequently processed by a single proteolytic cleavage prior to release as a two-chain molecule. Because of internal homology, the FVIII domain structure can be represented as A1–A2–B–A3–C1–C2. The B domain is not necessary for procoagulant function, as B-domain-deleted FVIII has full activity. The B-domain sequences of FV and FVIII share little sequence identity, although their lengths are similar,

and both are heavily glycosylated. It is likely that the glycosylated B domain functions in promoting endoplasmic reticulum/Golgi transport since deficiency of LMAN1 (also known as ERGIC 53), a mannose-binding protein, results in combined deficiency of FV and FVIII. FVIII also has three short acidic interdomain peptides (a1, a2 and a3) that are closely implicated in FVIII function.

FVIII is the essential procofactor for activation of FX by FIXa in the tenase complex. It has no function until proteolysed to FVIIIa by thrombin or FXa at Arg391-Ser392 (Arg372-Ser373) and Arg1708-Ser1709 (Arg1689-Ser1690). FVIIIa is directly inactivated by APC cleavage at Arg355-Ser356 (Arg336-Ser337) and Arg581-Ser582 (Arg562-Ser563); however, functional activity of FVIIIa also decays rapidly by dissociation of the A2 subunit from FVIIIa.

Factor V

The mature protein product is 2196 amino acids in length after cleavage of a 28-amino acid signal peptide. The structure of FV, like that of FVIII, can be represented as A1–A2–B–A3–C1–C2. In contrast with FVIII, the B domain is required for full procoagulant function, and it circulates in a single chain form. FV also differs from FVIII, in that it lacks the three short acidic interdomain peptides (a1, a2 and a3) implicated in FVIII function.

FV is the procofactor for the activation of prothrombin by FXa. It has no cofactor activity until proteolysed by thrombin or FXa at Arg737-Ser738 (Arg709-Ser710), Arg1046-Thr1047 (Arg1018-Thr1019) and Arg1573-Ser1574 (Arg1545-Ser1547). FVa is inactivated by APC through cleavages at Arg534-Gly535 (Arg506-Gly507) and Arg1793-Leu1794 (Arg1765-Leu1766). The initial (and rate-limiting) cleavage is at Arg534 (Arg506). This is the site of the variant in FV Leiden (FV Arg534Gln (Arg506Gln)) that is resistant to APC, leading to the most common form of familial thrombophilia.

Fibrinogen

The fibrinogen gene cluster is located on chromosome 4q32 in the order β–α–γ, with β transcribed in the opposite direction to α and γ. The three chains of fibrinogen are disulfide cross-linked and folded together in an intricate manner. The overall structure of fibrinogen is a symmetrical dimer, $\alpha_2\beta_2\gamma_2$. Viewed by electron microscopy, the molecule is trinodular, with the outer two globular domains (fragments D) containing the C-termini of all three chains connected to the central globular domain (fragment E), which contains the N-termini of all six chains tethered together by disulfide bonds. Coiled regions, forming α-helical ropes, connect the lateral and central globular domains.

Polymerisation of fibrinogen occurs when thrombin cleaves two short, negatively charged fibrinopeptides A and B from the N-termini of the α- and β-chains, respectively. This reveals new N-terminal sequences in the fragment E region (called knobs) that fit into holes in the fragment D regions. Polymerisation then occurs spontaneously in a staggered half-overlap array, which can elongate indefinitely in either direction. Electron microscopy studies have been useful for resolving the molecular architecture of fibrillar fibrin formation, but from 1997, elegant crystal structures of parts of the fibrin(ogen) molecule were successfully produced in a number of laboratories. These structures reveal the mechanisms of fibrin polymerisation.

Prothrombin

The mRNA encodes a preproleader sequence similar to that found in other vitamin K-dependent proteins, followed by a GLA domain, two kringle domains, an activation peptide and a serine protease domain. FXa complexed with FVa activates prothrombin zymogen to thrombin on a phospholipid surface (prothrombinase) on cleavage of two peptide bonds. The first, between Arg314 and Thr315 (Arg271 and Thr272), releases the protease domain from the GLA and kringle domains; the second, between Arg363 and Ile364 (Arg320 and Ile321), generates the catalytic site of thrombin by a typical trypsin-like conformational rearrangement. Cleavage at Arg363 (Arg320) alone yields the protease (meizothrombin) that retains membrane-binding properties. Cleavage at Arg314 (Arg271) yields thrombin and severs covalent linkage with the N-terminal domain known as fragment 1.2. Fully cleaved thrombin is termed α-thrombin and is rapidly released from its site of production to participate in numerous haemostatic functions free in solution: acting as a procoagulant against many substrates including fibrinogen, FV, FVIII and FXI; acting in complex with TM as an anticoagulant against PC; and acting to activate cellular transmembrane protease-activated receptors (PARs). Thrombin also activates thrombin-activatable fibrinolytic inhibitor (TAFI), in complex with TM, inhibiting fibrinolysis.

Naturally occurring inhibitors of blood coagulation

In common with other defence mechanisms, such as those resulting in kinin release and complement activation, the blood coagulation process can be activated very rapidly when the need arises. This involves the generation of proteolytic enzymes, such as thrombin, that are potentially lethal if their action is not limited. For example, 10 mL of plasma can generate, in theory, sufficient thrombin to clot all of the fibrinogen in the body in 30 s. That it does not normally do so is due partly to the fact that the procoagulant response is most pronounced in the vicinity of the platelet plug forming at the point of vascular

injury, while elsewhere coagulation is inhibited by substances in plasma or on the vascular surface that exhibits anticoagulant activity.

Classification of physiological anticoagulants

Physiological anticoagulants fall into two main groups: those that inhibit the serine proteases of the coagulation cascade (both Kunitz-type and serpins) and those involved in destruction of the activated coagulation cofactors FVa and FVIIIa (components of the PC system). These inhibitors assume great physiological significance: relatively minor deficiencies (50–70% of average levels) of some of these inhibitors, such as might be found in individuals heterozygous for a genetic defect, are associated with an increased risk of thrombosis. Homozygous deficiencies are frequently either fatal in the first few years of life unless prophylactic replacement therapy is instituted or are not found in nature, suggesting early fetal loss.

In addition to the specific inhibitors, there are some other inhibitory mechanisms that do not fit into either of the above categories, one of these being the detoxifying property of the liver, which plays an important role in removal of activated clotting factors, both directly and after their combination with natural inhibitors. Furthermore, the removal of free thrombin occurs as a result of its adsorption onto fibrin or onto fibrin(ogen) degradation products. The latter, which cannot themselves take part in a clot, may interfere with normal fibrin polymerisation. Although less well-defined than the others, this group appears to be physiologically important, as qualitative defects of fibrinogen (dysfibrinogenaemias) that result in reduced thrombin binding may be associated with a thrombotic disorder (see Chapter 48).

Tissue factor pathway inhibitor: a Kunitz-type inhibitor

The various serpins found in blood (see below) probably play no physiological role in the inhibition of FVIIa in the initiating TF–FVIIa complex. Instead, the action of this serine protease–cofactor complex is modulated by TFPI, which is located on the endothelial cell surface, within platelets, in plasma and on monocytes. Two alternatively spliced isoforms of TFPI (α and β) have been identified in humans that differ in their domain structure and mechanism of cell-surface binding. Both directly inhibit FXa and in a FXa-dependent manner inhibit FVIIa by forming a quaternary complex of TFPI–FXa–TF–FVIIa.

TFPIα has an acidic N-terminal region followed by three Kunitz-type protease inhibitory domains (K1–K3) and a basic C-terminal region. K2 is responsible for binding and direct inhibition of FXa and K1 inhibits FVIIa in an FXa-dependent manner. K3 and the C-terminal region do not directly inhibit proteolysis, but they are necessary for rapid inhibition of FXa by K2. K3 binds protein S (PS) and reduces the Ki for FXa inhibition by TFPI approximately 10-fold.

TFPIβ has the N-terminal acidic region followed by K1 and K2 but lacks K3 and the C-terminal region of TFPIα. Instead, it has a different C-terminal region encoding a glycosylphosphatidylinositol (GPI) attachment sequence that anchors the protein directly to the cell surface.

Most TFPI in the vasculature (~85%) is associated with endothelial cells, particularly in the microcirculation, with a smaller amount (5–10%) being found in platelets and the rest in plasma. A twofold to fourfold increase in plasma TFPI following infusion of heparin suggests a pool of TFPIα is associated with endothelial GAGS via the basic C-terminal region; however, this pool of endothelial associated TFPI has been difficult to characterise. TFPIα is the only isoform found in platelets, whereas endothelial cells express both TFPIα and TFPIβ. TFPIα is localised to intracellular granules and is released after cellular stimulation by thrombin. The majority (80%) of circulating plasma TFPI is associated with lipoproteins and has undergone variable amounts of proteolytic degradation within its C-terminal region and as a result has reduced anticoagulant activity and is unlikely to play an important role as an *in vivo* inhibitor of blood coagulation. Plasma TFPIα accounts for approximately 20% of total plasma TFPI and circulates in complex with either FV or PS. Recent evidence suggests that TFPIα circulates in complex with PS through binding to K3, and this enhances inhibition of the TF pathway primarily by localising TFPI to the membrane surface. In addition, TFPI binds a form of activated FV that retains the acidic region of the B-domain. This interaction results in reduced FV activation and inhibition of prothrombinase activity.

Disruption of the murine *Tfpi* gene is incompatible with normal development, suggesting that a deficiency of functional *TFPI* might lead to clinical thrombosis. However, as the bulk of intravascular TFPI is associated with the endothelial cell surface, simple measurement of plasma TFPI levels is not a clear indicator of a deficiency state. Moderately low plasma levels can occur in DIC, septicaemia and following major surgery, possibly due to increased utilisation, but heparin infusion increases plasma TFPI in such patients. Whether congenital deficiency of TFPI is a risk factor for thrombosis remains uncertain: after several negative studies in this area, recently a case–control study found a relationship between low levels of free plasma TFPI (non-lipoprotein bound) and enhanced risk of deep vein thrombosis and myocardial infarction. Anti-TFPI antibodies are currently in clinical trials for the treatment of haemophilia. The principle is that reducing blockage of FXa can rebalance the defect due to loss of FVIII or FIX, whether this is due to a deficiency or an inhibitor.

Serine protease inhibitors (serpins) and heparin

Human plasma contains at least seven inhibitors of serine protease coagulation factors. Apart from α_2-macroglobulin, all are single-chain serpins of molecular mass 40–65 kDa.

Specificity is imparted by their tertiary structure, which engenders high affinity for a defined substrate or small range of substrates. Of this group, only AT and heparin cofactor II (HCII) assume haemostatic significance, acting predominantly on proteases generated late in the coagulation cascade (i.e. thrombin and FXa). A deficiency of any of the other serpins (although sometimes giving rise to a clinical disorder due to the failure of neutralisation of a serine protease not involved in coagulation pathways) is asymptomatic in terms of haemostasis.

Several serpins contain GAG-binding sites that, particularly in the case of AT and HCII, but to a lesser extent for PAI-1 and APC inhibitor, greatly enhance the rate of interaction with (although not the affinity for) their specific protease(s). Since a major source of heparin-like material is heparan sulfate on the endothelial cell surface, another possible function of the heparin-binding site of these inhibitors (especially AT and HCII) might be to allow them to exert a general and constitutive anticoagulant effect on intact vessels; this may also act to prevent extension of a procoagulant response beyond an area of damaged endothelium.

Antithrombin

AT (formerly ATIII) is a 58-kDa serpin that is synthesised principally in the liver, with a high plasma concentration of 2.4 μmol/L. The turnover of AT *in vivo* is complex, with a rapid initial clearance ($t_{½}$ 10 min) due to equilibration with endothelial cell-bound AT, a slower clearance ($t_{½}$ 3 hours) due to equilibration with the extravascular compartment and a much slower linear phase with an overall $t_{½}$ of 90 hours.

Disruption of the murine AT gene results in death of all embryos shortly before birth due to severe cardiac and hepatic thrombosis. In humans, homozygous null variants are presumed lethal as they are only observed in the heterozygous state. Deficiencies of AT characterised by the presence of a dysfunctional protein are associated with heritable thrombosis.

AT forms a stable complex with several serine protease coagulation factors, predominantly thrombin and FXa, but also to some extent FIXa, FXIa, FXIIIa and kallikrein. Initially, the serine at the active centre of the protease cleaves a peptide bond (involving Arg425 (Arg393)) in the 'reactive centre loop' near the C-terminus of AT: a conformational change in the serpin ensues, resulting in irreversible trapping of the protease against the inhibitor. The inactivated enzyme–serpin complex is then cleared rapidly. Complex formation is progressive and only with thrombin and FXa is it rapid enough to be of physiological significance. In purified *in vitro* systems, the $t_{½}$ is around 30 s for free thrombin, 90 s for FXa and 10–25 min for the other enzymes. In each case, the inhibitor–protease complex is rapidly cleared ($t_{1/2}$ 3 min) from the circulation by the liver.

Heparin, without altering the stoichiometry, induces a more than 2000-fold increase in the rate of thrombin inactivation by AT, such that its action becomes almost instantaneous ($t_{½}$ < 0.01 s). The unique mechanism of this activation involves release of the reactive centre loop from partial insertion in the β-sheet core of AT following heparin binding. Heparin also strongly enhances AT neutralisation of FXa and, to a lesser extent, FIXa, particularly in the presence of Ca^{2+}. The enhancement of AT inhibition of thrombin and FXa by heparin is the basis of its use as a therapeutic anticoagulant to prevent thromboembolism. At therapeutic concentrations, there is no significant effect on AT inhibition of FIXa and FXIa.

Heparin cofactor II

This 65-kDa serpin is present in plasma at the high concentration of 90 mg/L (1.2 μmol/L). It appears to be a specific inhibitor of thrombin and to have little or no anti-FXa activity. The rate of thrombin neutralisation by HCII is increased approximately 1000-fold by heparin, although because of its lower heparin affinity it requires 5–10 times more heparin than does AT. Interaction seems to depend largely on the high anionic charge of heparin and related GAGs. Details of the molecular mechanism of HCII interaction with thrombin have been elucidated by X-ray crystallography.

That HCII has some physiological significance is suggested by the fact that it falls in parallel with AT in DIC. However, as AT is in twofold molar excess over HCII, the latter cannot altogether compensate for a deficiency of AT, which, as stated above, is a well-established cause of a thrombotic tendency. Whether HCII deficiency leads to a similar clinical picture remains to be established, as few cases have yet been described and only occasionally has concomitant thrombotic disease been present. Mice with HCII knockout develop normally and do not show spontaneous thrombosis; however, they show an enhanced propensity to carotid occlusion after deliberate injury to the endothelium, corrected by infusion of purified HCII, suggesting that HCII has a role in prevention of arterial thrombosis.

Heparin and heparin-like substances in plasma

Although the term 'heparin' implies a single compound, it in fact refers to a heterogeneous mixture of sulfated polysaccharides that are all members of the GAG group. The major pharmaceutical source is porcine intestinal mucosa. By virtue of its strong positive charge, heparin combines non-specifically with a number of cationic proteins such as albumin and reacts in a highly specific way with β-lipoproteins, fibrinogen, HCII and AT. It also mobilises platelet factor (PF)4 and TFPI, which are bound to GAGs on the surface of endothelial cells, and releases lipoprotein lipase into plasma.

Unfractionated heparin (UFH) is an extremely heterogeneous polymer, being composed of between 10 and 100 saccharide units. Only about one-quarter of the polysaccharides has any anticoagulant activity *in vitro*. The pharmaceutical properties of heparin are unpredictable, because of its heterogeneity, with marked variation

in pharmacokinetics and side-effects. Apart from bleeding, the most serious side effect is heparin-induced thrombocytopenia (see Chapter 48). The realisation that the anticoagulant action of UFH was mostly delivered by short pentasaccharides has led to the use of more efficient fractionation procedures (including cleavage by nitrous acid, heparinase and oxidising agents), yielding a number of low-molecular-weight heparin (LMWH) preparations. LMWHs contain 10–20 saccharide units (4–6 kDa) and because of their more uniform composition have much better pharmacokinetics and fewer side-effects. Fondaparinux is a synthetic pentasaccharide that is now in therapeutic use and offers increased half-life compared with larger LMWHs.

UFH and the various LMWHs differ in their affinities for the plasma factors with which they interact and in the specific serine proteases that they inhibit. This, together with the timing of blood samples in relation to dosage interval, is critically important in laboratory monitoring of the therapeutic effect (see Chapter 47). The major anticoagulant activity of both UFH and LMWH is attributed to the pentasaccharide sequence containing at its centre a 2,3,6-(SO_3) trisulfated glucosamine group. It is this that binds to lysine or tryptophan residues in AT in the region of the D helix, inducing the conformational change in the inhibitor. A second anticoagulant action of UFH (at least 18 saccharide units are required) involves direct binding between thrombin (but not FXa or other proteases) and a heparin sequence adjacent to the pentasaccharide. This brings thrombin and AT into close proximity and is necessary for the inhibitory activity. As many chains in an LMWH have fewer than 18 saccharide units, they have less antithrombin action compared with their inhibition of FXa. There is no detectable heparin in normal plasma. However, endogenous heparin-like molecules (e.g. dermatan sulfate and heparan sulfate) are present on the surface of endothelial cells and, by enhancing the action of AT and HCII, these would have antithrombotic effects. Such mechanisms seem to be of clinical importance, as recurrent thromboses have been reported in association with several dysfunctional AT molecules that inhibit thrombin normally in the absence of heparin, but which do not show enhanced reactivity in its presence, presumably due to defects at the heparin-binding sites. As this leads to heparin resistance, alternative therapeutic anticoagulants or AT replacement therapy is required. At points of vascular injury, local accumulation of activated platelets provides a source of heparin-neutralising activity that could overcome the anticoagulant effects of endothelial cell-bound heparinoids and permit the procoagulant response to proceed.

Protein Z and protein-Z-dependent inhibitor

PZ is a 62-kDa vitamin K-dependent plasma protein that serves as a cofactor for the inhibition of FXa by PZ-dependent protease inhibitor (ZPI) a 72-kDa member of the serpin superfamily.

The organisation of the PZ gene and the structure of the molecule are very similar to those of coagulation factors FVII, FIX, FX and PC; however, the PZ 'serine protease' domain lacks the canonical active-site His and Ser residues and therefore cannot function as a protease. PZ circulates in plasma in a complex with ZPI. Inhibition of FXa by ZPI in the presence of phospholipid and Ca^{2+} ions is enhanced 1000-fold by PZ, but ZPI also inhibits FXIa in a process that does not require PZ, phospholipid or Ca^{2+}. ZPI activity is consumed during coagulation through proteolysis mediated by FXa (with PZ) and FXIa. PZ may serve to dampen the procoagulant response *in vivo* as PZ deficiency dramatically increases the severity of the prothrombotic phenotype of FV Leiden mice. Studies to determine the potential roles of PZ and ZPI deficiency in human thrombosis are in progress.

α_1-Antitrypsin

This is a serpin whose primary targets are pancreatic and leucocyte elastases. In coagulation, the major inhibitory activity is directed against FXIa and FXa, although the chief physiological inhibitor of FXIa is probably protease nexin 2. It has little effect on overall thrombin inhibition, and a straightforward deficiency of α_1-antitrypsin is not associated with hypercoagulability, although it does cause lung and liver disease.

An abnormal molecular form of α_1-antitrypsin in which there is a Met→Arg substitution at the active centre (antitrypsin Pittsburgh; Met358Arg) has been described, resulting in a higher affinity for thrombin. At a plasma concentration of 25 μmol/L, the variant circulates at a 10-fold higher level than AT and gives rise to a clinical bleeding tendency. However, it is unaffected by heparin.

Protease nexin 2

This is a Kunitz-type serine protease inhibitor that is found in the α-granules of platelets. Normally, there is very little in plasma, but it is released at sites of platelet activation, following which the single Kunitz domain enters into a tightly bound inhibitory complex with FXIa. It is one of several isoforms of Alzheimer β-amyloid protein precursor present in platelets. Deficiency of protease nexin 2 has not been described.

C1-esterase inhibitor

The primary target of this serpin is the activated form of the first component of complement, but it also contributes in a minor way to the neutralisation of FXIa and plasmin. Deficiency of the C1-esterase inhibitor, although of no haemostatic consequence, causes angioneurotic oedema, the characteristic lesions of which may sometimes be confused with haematomas.

α_2-Antiplasmin

This serpin is the principal inhibitor of the fibrinolytic enzyme plasmin. It also has weak activity against several coagulation proteases, especially the contact factors. However, any

anticoagulant action against proteases late in the coagulation cascade (e.g. FXa) is only apparent at concentrations well in excess of those in normal plasma. Its mechanism of action and clinical importance are discussed further in the section on fibrinolysis.

α_2-Macroglobulin

α_2-Macroglobulin is composed of four identical chains and has a molecular mass of 740 kDa. It is not a member of the serpin superfamily, and its effects are not restricted to serine proteases. It binds to coagulation factors at a site away from the active site, the interaction involving the formation of a bond between cysteine and glutamate residues in the inhibitor and a lysine residue in the protease. Inhibition is produced by stearic hindrance rather than by active site inactivation, and indeed the proteases retain some esterolytic and amidolytic activity, particularly against small peptides, a fact that should be borne in mind when using chromogenic substrates to assay coagulation inhibitors. It is responsible for approximately 50, 20 and 10% of the inhibition of kallikrein, thrombin and FXa, respectively.

Deficiency of α_2-macroglobulin is not associated with a thrombotic tendency. However, it is an acute-phase reactant, and it is possible that, when elevated under conditions of stress or when the other major antithrombins or antiplasmins are overwhelmed, it might become a significant inhibitor of coagulation or fibrinolysis. Moreover, it has been suggested that the increased level of α_2-macroglobulin that exists in children (150–200% of adult values) may compensate for a low level of AT and explain why thrombotic episodes do not usually occur before puberty in congenitally AT-deficient patients.

The protein C pathway: inhibition of cofactors FVa and FVIIIa

The activated forms of coagulation cofactors FV and FVIII (FVa and FVIIIa) are potent procoagulants that enormously enhance the activity of serine protease factors in the tenase and prothrombinase complexes (see above). It is not unexpected that these cofactors should be subject to a negative feedback mechanism that limits their procoagulant activity. This is achieved by a complex series of reactions collectively referred to as the protein C pathway. Four key factors are now known to be involved, and their role in the haemostatic network is shown in Figure 39.4.

Protein C

This vitamin-K-dependent serine protease has an identical modular composition to the procoagulant factors FVII, FIX and FX. It circulates in blood as a two-chain disulfide-linked molecule. During biosynthesis, the primary translation product is proteolytically processed, releasing the dipeptide Lys198Arg199 (Lys156Arg157). In order to exert its anticoagulant effect, PC must first be activated to APC. This is achieved by the action of thrombin, which cleaves the heavy chain to release a 12-residue Gly200–Arg211 (Gly158–Arg169) activation peptide, revealing the active site by the usual chymotrypsin-like mechanism. Thrombin activation of PC is slow in free solution but is markedly accelerated by specific endothelial cell receptors for both thrombin (TM) and PC (EPCR), which coordinate the assembly of a membrane complex for PC activation.

APC interacts with PS bound to the phospholipid surface of activated platelets, enhancing the anticoagulant activity of APC (see below) against FVa and FVIIIa. These procoagulant cofactors are inactivated by APC on the platelet surface by specific cleavage in their A domains, terminating the activity of the tenase and prothrombinase complex by disrupting their binding sites for FIXa and FXa, respectively. In the absence of PS, this reaction is inefficient. The most common form of familial thrombosis is FV Leiden, which is caused by a variant of Arg534 to Gln (Arg506 to Gln), which makes the molecule resistant to cleavage by APC. So common is this variant (5–8% of Caucasians), that it is likely to have a beneficial effect on bleeding at delivery balancing the mild prothrombotic effect. It appears that APC inactivation of FVa is a prerequisite for efficient inactivation of FVIIIa *in vivo*, as FVIIIa inactivation is also impaired in these patients, although there is no abnormality in the FVIII molecule. Since the discovery of FV Leiden, other less common FV variants have been found with a similar phenotype. None of the variants reported in the FVIII molecule has been shown to cause a similar prothrombotic phenotype. PC is synthesised in the liver, and being vitamin K-dependent, is often low in the newborn population. Even though its substrates (FVa and FVIIIa) are normal at birth, this deficiency is compensated for by the reduction in plasma of the vitamin K-dependent procoagulant factors (prothrombin, FVII, FIX and FX). Because warfarin treatment initially decreases PC levels faster than FVII, FIX, FX and prothrombin levels, it can paradoxically increase the procoagulant tendency when anticoagulant treatment is first begun. This is why warfarin is not used in the initial anticoagulant treatment of acute thrombosis. Disruption of the murine PC gene results in lethal perinatal consumptive coagulopathy, whereas in humans, homozygous PC deficiency is associated with lethal purpura fulminans (in the absence of PC replacement therapy) and heterozygous individuals have a high risk of venous thrombosis. There is increasing appreciation of the role of the PC pathway in regulation of inflammation and the concept of signalling mechanisms allowing 'cross-talk' between the haemostatic and inflammatory networks. This is well-demonstrated by the protective effect of administration of APC concentrate to animals with induced sepsis. However, the concentrate has not proven to be quite so

efficacious in human studies. Recently, variant recombinant PC molecules have been produced that appear to have either anticoagulant or anti-inflammatory effects independent of each other.

Thrombomodulin

TM is an integral transmembrane receptor found on endothelial cells in virtually all body tissues. It appears to be absent in the brain vasculature and in hepatic sinusoids and lymph node venules. TM is essential for normal fetal development as shown by mouse knockout studies, although $TM^{+/-}$ heterozygous mice appear to be completely healthy.

The protein has an extracellular region composed of an N-terminal lectin-like or CLECT domain, six EGF-like domains, a transmembrane sequence and a short cytoplasmic region. TM forms a complex with thrombin (via the protease's anion-binding exosite I and TM's EGF-like domains 4–6), preventing binding of the protease to its various procoagulant substrates (fibrinogen, FV, FVIII, FXIII and protease-activated receptors involved in platelet aggregation). TM also plays a part in binding of PC zymogen and, after formation of the TM–thrombin complex on the cell surface, there is a 20,000-fold increase in the rate of activation of PC, so that thrombin effectively becomes an anticoagulant. Binding of PC to TM is also enhanced by EPCR.

The TM–thrombin complex is short-lived, being endocytosed by endothelial cells, where the thrombin is taken up and degraded by lysosomes while TM re-circulates to the cell membrane.

Endothelial protein C receptor

EPCR is a transmembrane receptor on endothelial cells that binds PC and promotes its activation by the thrombin–TM complex. The extracellular portion of EPCR is related to the MHC class I/CD1 proteins; however, it lacks the α3 domain found in the extracellular portion of most of the family: there is good evidence that the α1 and α2 extracellular domains of EPCR interact with the GLA domain of PC. EPCR may function by localising the PC molecule to the endothelial cell surface, moving laterally on the cell surface to locate a thrombin–TM complex, then presenting the PC molecule optimally for thrombin cleavage to form APC.

The essential role of EPCR was demonstrated by targeted deletion of the murine *EPCR* gene, resulting in early fetal death, with evidence of thrombosis at the maternal–fetal interface. Abrogation of EPCR function *in vitro* reduces the rate of PC activation by thrombin–TM. In contrast, inhibition of EPCR action by a specific anti-EPCR monoclonal antibody in baboons given thrombin resulted in reduced APC levels amidst evidence of excessive coagulopathy. Blockade of EPCR on challenge with *Escherichia coli* also led to worse outcomes in an animal model of sepsis, whereas administration of APC improved survival in experimental sepsis.

Protein S

PS is a single-chain vitamin K-dependent glycoprotein chiefly synthesised in the liver by endothelial cells. Unlike the other vitamin K-dependent coagulation proteins, it is not a serine protease, having two C-terminal LamG domains rather than a serine protease domain, and the N-terminal GLA domain is followed by four EGF-like domains rather than the two usually seen in the serine proteases (see Figure 39.8). About 40% of the PS in plasma is in the free form, whereas the remaining 60% is associated in a complex with C4b-binding protein (C4bBP). Both forms bind strongly via the PS GLA domain to negatively charged phospholipids exposed on the surface of activated platelets. Free PS (although having no strong inhibitory effect on FVa or FVIIIa itself) forms a Ca^{2+}-dependent complex with APC, helping to orient the APC active site above the phospholipid surface and enhancing its anticoagulant activity against both proteins. However, C4bBP-bound PS does not enhance PC function against FVa. Free thrombin cleaves PS between the GLA domain and EGF1, removing its ability to bind to both phospholipid and PC and thereby abolishing its PC cofactor activity. Although PS was mainly considered to function as a cofactor in the activity of APC, it is now evident that PS also has an important role in the action of TFPIα in inhibiting TF-FVIIa-FXa and FXa.

Protein C inhibitors

As with other serine proteases, APC is subject to inhibition by serpins (half-life of APC in plasma is 15–20 min), including APC inhibitor (PCI), PAI-1 and α_1-antitrypsin. The relative contributions of these *in vivo* are difficult to assess. PCI slowly but progressively blocks the action of APC (and, to a much lesser extent, thrombin and FXa), in each case forming a 1:1 complex; in addition, PCI action is enhanced 20-fold by heparin and more weakly by other GAGs, but the physiological significance of this is uncertain. Despite the name, disruption of the murine *PCI* gene leads not to coagulopathy, but to infertility due to lack of inhibition of another protease target of the serpin, the sperm protein acrosin.

Fibrinolysis

It is widely acknowledged that the principal functions of the fibrinolytic system are to ensure that excess fibrin deposition is either prevented or rapidly removed. This is vital for ensuring that a localised procoagulant response is achieved without compromising blood circulation generally. Following re-establishment of

haemostasis, the fibrin mesh is removed during wound healing. The system of profibrinolytic and antifibrinolytic factors that has evolved to meet these requirements is closely coupled to that, which results in fibrin clot formation. Fibrinolysis is essentially a localised, surface-bound phenomenon, with most events being catalysed by the presence of cross-linked fibrin itself, i.e. 'fibrin orchestrates its own destruction'. For this reason, the assays of fibrinolytic factors carried out in the soluble phase, in particular in systemic blood, may be misleading and should be interpreted with great caution.

Components of the fibrinolytic system

These include plasminogen (PLG) and plasmin, several endogenous (tissue or plasma derived) or exogenous (e.g. bacterial or venom derived) PLG activators and a number of inhibitors of plasmin or of the PLG activators. Both endogenous and exogenous fibrinolytic factors have been used clinically to treat venous and arterial thrombosis, with varying degrees of success (see Chapters 49 and 50).

Plasminogen and plasmin

PLG is a single-chain glycoprotein zymogen of the serine protease plasmin, which carries out the enzymatic degradation of cross-linked fibrin. Besides its active site serine, plasmin contains five kringle domains, four of which have a lysine-binding site, through which the molecule interacts with lysine residues in its substrates (e.g. fibrin), its activators (e.g. tissue PLG activator and urinary PLG activator) and its inhibitors (principally PLG activation inhibitor type 1). In its native form, it has a glutamic acid residue at its N-terminus and is known as Glu-PLG. Conversion of PLG to plasmin can proceed via two routes. Most PLG activators (see below) cleave the Arg580–Val581 (Arg561–Val562) bond to form Glu-plasmin, a disulfide-linked two-chain molecule. The heavy chain is derived from the N-terminal region and bears the lysine-binding sites, whereas the C-terminal light chain contains the serine active centre. Glu-plasmin, despite being a serine protease, is functionally ineffective, as its lysine-binding sites remain masked. It is converted autocatalytically to Lys-plasmin by N-terminal cleavage, chiefly between Lys96 and Lys97 (Lys76 and Lys77), which exposes the lysine-binding sites and thus markedly enhances its interaction with fibrin.

Both Glu- and Lys-plasmin also attack the same Lys96–Lys97 (Lys76–Lys77) bond in Glu-PLG to form the zymogen Lys-PLG. This binds to fibrin before activation to the protease, bringing it into close proximity with the physiological PLG activators (which also bind to fibrin) that convert it to Lys-plasmin. As a consequence, the conversion of PLG to plasmin by tPA is enhanced by two to three orders of magnitude; this serves to localise the fibrinolytic response to the fibrin clot, where plasmin is to some extent protected from the effects of circulating antiplasmins, which (as indicated below) would otherwise neutralise plasmin extremely rapidly (<50 ms). The fact that Lys-PLG is potentially a much more effective agent in fibrinolysis than Glu-PLG is reflected in its half-life, which is around 20 hours compared with 50 hours for the latter.

Action of plasmin on fibrin and fibrinogen

Plasmin can hydrolyse a variety of substrates, including FV and FVIII, but its major physiological targets are fibrin and fibrinogen, which are split progressively into a heterogeneous mixture of small soluble peptides (plasmin attacks at least 50 cleavage sites in fibrinogen) known collectively as fibrin degradation products (FDPs).

The first stage in the proteolysis of fibrinogen involves the removal of several small peptides (fragments A, B and C) from the C-terminus of the A α-chains, each involving cleavage after a lysine residue. This is rapidly followed by removal of the first 42 amino acids from the N-terminal end of the B β-chain (the Bβ1–42 fragment). The large residual portion, which is known as fragment X, and which still contains fibrinopeptide A, remains thrombin-clottable and will agglutinate some species of *Staphylococcus* spp. Assay of the Bβ1–42 fragment released from fibrinogen by plasmin gives a sensitive index of fibrinogenolytic activity.

Asymmetrical digestion of all three pairs of chains of fibrin or fibrinogen then occurs with the release of the D fragment, in which the chains remained linked by disulfide bonds. The residue, known as fragment Y, is again attacked by plasmin, cleaving a second fragment D and leaving the disulfide-linked N-terminal ends of all six chains, which are referred to as fragment E. Fragments Y, D and E are not thrombin-clottable and do not agglutinate staphylococci. Their presence can be detected immunologically using specific antibodies, which provides a simple test for most FDPs, although carefully prepared serum must be used to prevent cross-reactivity of the antibody with fibrinogen in plasma. These assays detect the degradation products of both fibrin and fibrinogen indiscriminately.

Following thrombin generation and consequent activation of FXIII, intermolecular or intramolecular transamidation of the fibrin α- or β-chains by FXIIIa occurs, and then the action of plasmin yields characteristic D-dimer, D-dimer–E fragments and oligomers of fragments X and Y (collectively known as cross-linked FDP or XDP), in addition to X, Y, D and E. These XDPs can be detected very simply using monoclonal antibodies. Because the monoclonal antibodies to XDPs do not cross-react with fibrinogen, they can be detected directly in citrated plasma. The presence of D-dimers in blood samples can be used in a clinical algorithm that predicts the likelihood of the presence of venous thrombosis (see Chapter 49).

Furthermore, plasmin-induced cleavage of the N-terminal end of the β-chain of fibrin (the Bβ1–14 fibrinopeptide B fragment having been removed by thrombin) produces a β15–42 fragment, the detection of which indicates fibrin (as opposed to fibrinogen) degradation. Consequently, assays for the Bβ1–42 and β15–42 fragments used in combination may be clinically useful by indicating whether fibrinogen or fibrin has been degraded, and thus whether fibrinolytic activity is primary or secondary to fibrin formation. However, clinically, FDP assays are used to detect DIC, when mixed fibrin/fibrinogen degradation products appear in the circulation.

Plasminogen activators

Tissue plasminogen activator

tPA is a serine protease secreted by endothelial cells. It is not synthesised by the liver or kidney, but is found in most extravascular body fluids, including saliva, milk, bile, cerebrospinal fluid and urine. Intravascular tPA is quickly cleared by the liver or inactivated by the fast-acting tPA inhibitor (see below), the half-life of tPA in plasma being approximately 2 min. The resting level of tPA in plasma is around 70 pmol/L, most of which is in an inactive complex with tPA inhibitors (see below).

Several physical and biochemical stimuli, including venous occlusion, strenuous exercise, thrombin, adrenaline and vasopressin or its analogues such as desmopressin (see Chapter 41), markedly increase the rate of tPA release, although its biological activity remains negligible until it becomes bound to fibrin, whereupon its affinity for and action upon PLG is greatly potentiated. The activity of tPA is further enhanced by plasmin itself, which cleaves tPA at Arg310-Ile311 (Arg275-Ile276) into a two-chain molecule, whose binding sites are exposed, thus enabling it to form a complex with PLG and fibrin more readily. The ability of venous occlusion to stimulate tPA release from endothelial cells forms the basis of a test of fibrinolytic activity known as the 'cuff test'.

Both single-chain tPA and the two-chain form possess very little serine protease activity until they bind to fibrin, whereupon tPA affinity for, and activation of, fibrin-bound PLG is increased at least 100-fold. The principal interactions involve binding between the second kringle domain of tPA and lysine residues on the α- and β-chains of fibrin (in particular Lys157 on the α-chain of partly degraded fibrin). This association enhances cleavage of the Arg580–Val581 (Arg561–Val562) bond in adjacent PLG molecules, forming active plasmin.

Urinary plasminogen activator

So called because it was first extracted from urine, uPA is synthesised chiefly by the tubules and collecting ducts in the kidney and by fibroblast-like cells in the gastrointestinal tract. It is a serine protease secreted as an inactive single-chain zymogen (prourokinase) that is cleaved by activators in plasma (including kallikrein and plasmin) at Lys178–Ile179 (Lys158–Ile159) to produce active two-chain uPA. The protease activity of uPA is associated with the heavy chain, which may dissociate from the light chain carrying the PLG-binding site. The isolated heavy chains, which are also known as low-molecular-weight urokinase, are therefore poorer PLG activators than the two-chain form.

uPA cleaves the same Arg580–Val581 (Arg561–Val562) bond in PLG as tPA. Although uPA contains a kringle domain, this does not impart high affinity for fibrin, and it binds instead (via its EGF-like domain) to its cell-associated receptor. Thus, it has been proposed that uPA may be preferentially involved in cellular events (such as differentiation and mitogenesis) rather than with dissolution of fibrin clots.

Exogenous plasminogen activators

These are derived from non-human sources, including animals (e.g. vampire bat saliva and some snake venoms) and certain plants and microorganisms. The best known of these is streptokinase (SK), which is derived from some strains of β-haemolytic streptococci, and which has for many years been used, with moderate success, as a fibrinolytic agent for the treatment of life-threatening thrombotic states. Despite its name, SK is a non-enzymatic polypeptide that forms a stable complex with PLG, as a result of which the latter undergoes a conformational change, unmasking its serine-active centre. The 'plasmin' that is formed remains associated with SK but can convert free PLG to plasmin.

Inhibitors of fibrinolysis

The plasmin-generating potential of plasma is sufficient to completely degrade all the fibrinogen in the body in a very short period of time. It is prevented from doing so by the PLG activator inhibitors or PAIs, most of which belong to the serpin family, and by a number of circulating inhibitors of plasmin itself (the antiplasmins).

Inhibitors of plasminogen activation

Plasminogen activator inhibitor type 1

PAI-1 is a fast-acting serpin inhibitor of tPA, uPA and, to a small extent, plasmin and is secreted by endothelial cells. It is also found in platelet α-granules. In plasma, it occurs in two forms: a functionally active 'free' form (that is stabilised by association with vitronectin) and as an inactive complex with tPA. Basal PAI-1 concentration in plasma is low at 0.5 nmol/L, of which at least 80% is in complex with tPA or uPA. It follows a diurnal

rhythm, with an early morning peak that is around twice that in the late afternoon, and its activity is also increased by heparin. Elevated levels of PAI-1 are associated with an increased incidence of venous and arterial thrombosis, perhaps as part of a multi-factorial process involving metabolic disturbance and obesity. While common variants in the *PAI-1* gene (*SERPINE1*) were previously thought to increase the risk of thrombosis, genome-wide association studies have not shown any convincing effect.

The three main profibrinolytic serine proteases (tPA, uPA and plasmin) all cleave the same bond Arg369–Met370 (Arg346–Met347) in the reactive centre loop of PAI-1 and are thus inhibited in the same way as described in the section on AT. PAI-1 binds non-covalently to fibrin, but although it can then complex with and inhibit fibrin-bound tPA (with most of the complexes remaining bound to the fibrin), it does so less effectively than with free tPA. Soluble tPA–PAI-1 complexes are rapidly removed by the liver ($t_{½}$ 4 min), as are uPA–PAI-1 complexes that have dissociated from fibrin.

Plasminogen activator inhibitor type 2

This serpin inhibitor of tPA is mainly produced by the placenta and may thus contribute to the inhibition of fibrinolysis that occurs during pregnancy. It is also synthesised in monocytes and epidermal cells but is not usually found in the plasma of non-pregnant subjects. It is detectable in plasma from about the eighth week of pregnancy, rising to a peak at around 33 weeks and falling only slowly after delivery, the half-life being around 24 hours. Paradoxically, levels are often low in pre-eclampsia due to placental insufficiency. The inhibitory action of PAI-2 involves its Arg380–Thr381 residues, and it is more effective against uPA than tPA, although for both the potency is at least 10-fold less than that of PAI-1.

Similarly, other protease inhibitors such as α_1-antitrypsin, C1-esterase inhibitor, α_2-antiplasmin (see below), α_2-macroglobulin and protease nexin 1 (one of a group of cell membrane-bound heparin-potentiated serpins) also neutralise tPA, but at a rate that is probably too slow to be of physiological significance. However, α_2-macroglobulin is thought to be the major inhibitor of the SK–PLG complex.

Inhibitors of plasmin

As they do with thrombin and tPA, a number of the broad-spectrum inhibitors contribute to neutralisation of plasmin. By far, the most potent plasmin inhibitor is the serpin α_2-antiplasmin, a single-chain glycoprotein synthesised by the liver, which has a half-life of about 60 hours and shows considerable sequence identity with AT and α_1-antitrypsin. Its physiological importance is supported by the fact that a congenital deficiency (known as Miyasato disease) is associated with a clinically significant bleeding disorder due to uncontrolled fibrinolytic activity, and that levels are reduced in DIC and during thrombolytic therapy.

α_2-Antiplasmin

This serpin is the predominant plasmin inhibitor. It forms a stable complex with plasmin, in which the protease is completely inactivated. The reaction appears to involve the cleavage by plasmin of a specific Leu–Met bond in the inhibitor, exposing the reactive loop Arg376–Met377 (Arg364–Met365) peptide bond to the serine-active centre on the light chain of plasmin. Plasmin-modified α_2-antiplasmin can also bind to native Glu-PLG and to fibrin, the latter reaction being mediated by FXIIIa-mediated cross-linking. Thus, in addition to inactivating preformed plasmin, α_2-antiplasmin retards fibrinolysis by reducing PLG activation and by 'masking' the lysine-binding sites through which plasmin(ogen) interacts with fibrin. However, these subsidiary mechanisms for inhibiting fibrinolysis are to some extent overcome by any Lys-plasmin(ogen) present, which has a higher affinity than Glu-PLG for fibrin and is thus less susceptible to the action of α_2-antiplasmin.

In plasma (as opposed to on fibrin strands), although the concentration of PLG (~2 μmol/L) exceeds that of α_2–antiplasmin (~1 μmol/L), basal fibrinogenolytic activity is minimal because the tiny amounts of plasmin normally generated under physiological conditions are rapidly neutralised by the inhibitor. However, in certain pathological conditions (e.g. obstetric emergencies or snake bite) where extreme activation of fibrinolysis occurs, the latter may be swamped. Under these circumstances, other inhibitors, in particular α_2-macroglobulin and histidine-rich glycoprotein, may become clinically important. The action of α_2-macroglobulin on plasmin is similar to its effect on thrombin. Following the plasmin-induced cleavage of a specific Arg–Leu bond in the inhibitor, the latter forms a complex with the light chain of plasmin. The serine active site of plasmin is not involved, and the complex retains weak biological activity, albeit only briefly, until it is removed in the liver. Histidine-rich glycoprotein inhibits fibrinolysis by blocking the lysine-binding sites of PLG, thus preventing its interaction with fibrinogen.

Lipoprotein A

The protein portion of lipoprotein A is termed apo(a). It is synthesised in the liver and circulates in plasma. There is considerable structural homology with PLG, as it possesses both serine protease and kringle domains. It can compete with PLG for binding sites on fibrin(ogen) or tPA and may also increase PAI-1 expression, both actions potentially inhibiting fibrinolysis. That lipoprotein A has some clinical importance is indicated

by the finding that increased levels are associated with an increased incidence of thrombosis.

Thrombin-activatable fibrinolysis inhibitor

In the presence of thrombomodulin, thrombin activates carboxypeptidase B, also called TAFI, and TAFIa in turn inhibits fibrinolysis; this provides another link between coagulation and the fibrinolytic pathway. TAFI removes the C-terminal lysine residues formed by limited plasmin proteolysis of fibrin, removing the binding sites for PLG and tPA. Thus, the fibrin cofactor function in PLG activation is reduced, downregulating fibrinolysis. Mice with TAFI knockout have defective wound repair, and data from backcrossing against heterozygous PLG-deficient mice showed that TAFI modulates both fibrinolysis and cell migration *in vivo*.

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CHAPTER 40

The vascular function of platelets

40

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Introduction

Platelets circulate in the bloodstream in a quiescent state and undergo rapid activation at sites of vessel damage. To achieve this balance, endothelial cells constitutively release nitric oxide (NO) and generate prostacyclin to inhibit platelet activation through elevation of the cyclic nucleotides cGMP and cAMP. In addition, endothelial cells express the ecto-ADPase CD39, which metabolises ADP and ATP to adenosine, which inhibits platelet activation through the G protein-coupled adenosine A1 receptor. Under normal circumstances, platelets are unable to form stable contacts on endothelial cells, but this can occur on diseased or activated endothelial cells, or at sites of disturbed flow, including arterial bifurcations where plaques are commonly formed. Adhesion of platelets to endothelial cells promotes local inflammatory events that lead to endothelial cell activation and damage, and thrombo-inflammatory disorders such as atherosclerosis and deep vein thrombosis. Lesions in the endothelial layer expose subendothelial matrix proteins, particularly collagens and membrane surface-expressed tissue factor, which initiate haemostasis.

Platelets are now recognised to have a much wider physiological role than simply supporting haemostasis (Figure 40.1). Platelet-dense granules and α-granules are packed with a rich diversity of small molecules and proteins that play fundamental roles in many aspects of haemostasis, including vessel constriction, leucocyte recruitment and vessel repair, as well as in other pathways, including host defence. In addition, platelets release and support the formation of a variety of active lipid mediators including sphingosine 1-phosphate, thromboxane A_2 (TxA_2) and lysophosphatidic acid. The ability to generate such a cocktail of biological molecules may reflect the evolutionary relationship of the platelet to the haemocyte in invertebrates, which is involved in both the innate defence system and haemostasis. In mammals, two distinct sets of cells perform these functions, thrombocytes (or platelets) and leucocytes.

Platelets are a major therapeutic target in arterial thrombosis. However, careful consideration is required as to whether a patient should receive antiplatelet therapy and for how long, bearing in mind that such treatment always carries a risk of increased bleeding. The decision is based on the assessment of risk factors for arterial thrombosis, such as age, hypertension, smoking status and history of previous coronary artery or cerebrovascular disease, and the potential risk from excessive bleeding. It is now accepted that individuals judged to be at medium to high risk of thrombosis should receive some form of antiplatelet therapy (unless otherwise contraindicated), usually low-dose aspirin, and nearly always in combination with other treatments such as statins and blood-pressure-lowering drugs. In certain situations (e.g. newly inserted drug-eluting coronary artery stent, or recurrent events on a single antiplatelet agent), there is a role for using a $P2Y_{12}$ receptor antagonist such as clopidogrel or ticagrelor in combination with aspirin, but this is associated with an increased risk of bleeding. Stronger inhibitors of platelet function, namely blockers of the major platelet integrin αIIbβ3 (GPIIb-IIIa) such as eptifibatide, are used only in acute situations of thrombotic risk, for example in cases of unstable angina or angioplasty and stenting, because of their narrow therapeutic window.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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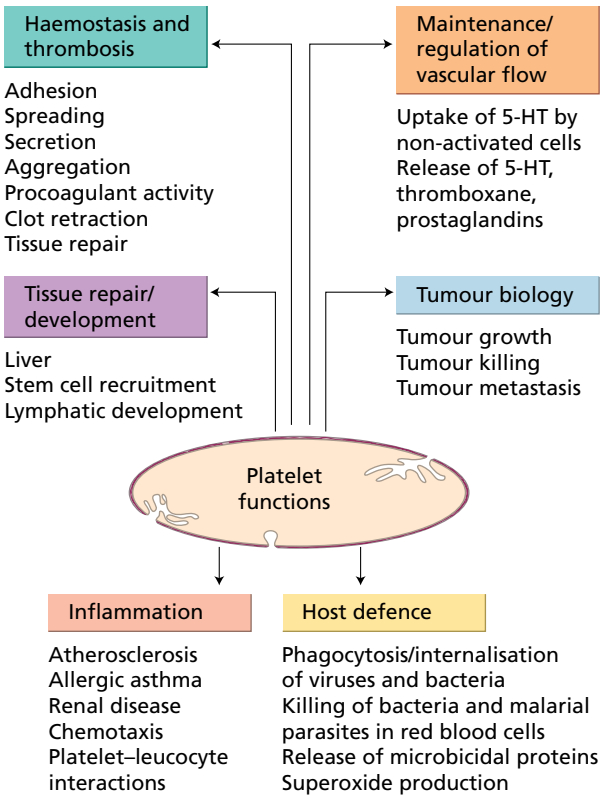

Figure 40.1 Functional roles of the platelet in the vasculature.

Platelet structure and organelles

Platelets are discoid in shape, with dimensions in the human of approximately 3.0×0.5 µm and a mean volume of 7–11 fL. This shape and small size enable platelets to be 'marginated' by red blood cells to the edge of the vessel, placing them in contact with vascular endothelial cells. The platelet count in humans is usually in the range $150–400 \times 10^9$/L. This count represents a considerable degree of redundancy for haemostasis, as major spontaneous bleeds are usually only seen when the platelet count falls below 20×10^9/L. Low platelet counts are also associated with increased risk of excessive bleeding during major trauma and surgery. For most forms of surgery (neurosurgery excluded) a platelet count of 50×10^9/L or above is acceptable to secure haemostasis during the procedure.

The discoid shape of the platelet is regulated by the platelet cytoskeleton, which consists of a spectrin-based membrane skeleton, circumferential bands of a single microtubule that lie beneath the plasma membrane, and a rigid actin filament network that fills the cytoplasm of the cell. The rigid structure is supported by two million copies of actin per platelet, of which approximately 40% in a circulating cell are assembled into actin polymers. These polymers connect with each other and with the cytosolic tail of the membrane glycoprotein (GP) Ibα via filamin in a lattice-like structure.

Platelets lack a nucleus and have a short lifespan of 10 days. They have a very limited ability to undergo protein synthesis as the result of residual mRNA that has been carried over from their precursor cell, the megakaryocyte. However, evidence suggests that activated platelets can synthesise a small number of key proteins (e.g. IL-1β) that may be of functional significance. The presence of low levels of platelet mRNA provides an opportunity to perform a limited number of genetic studies, although concern over contamination from nucleated cells, which express several orders of magnitude higher levels of mRNA, necessitates the need for confirmation of protein expression. Despite these concerns, the application of both genomic and proteomic approaches has given an unprecedented insight into the protein composition of the platelet; the challenge remains however to identify the function and interplay of the proteins, as many may be vestigial or have been carried over from the megakaryocyte.

Platelets contain four main types of storage granule: dense granules, α-granules, lysosomes and peroxisomes, and several mitochondria. There are between 5 and 9 dense granules in each platelet which contain high levels of ADP, ATP, polyphosphate, 5-hydroxtryptamine (5-HT: serotonin) and Ca^{2+}. There are approximately 80 α-granules per platelet and these contain a rich diversity of proteins and membrane receptors that support haemostasis, vascular repair, inflammation and host defence (Table 40.1).

Table 40.1 Platelet α-granule constituents. Examples of platelet α-granule constituents and their physiological roles are shown.

Physiological role	Constituent
Angiogenesis	VEGF-A, VEGF-C and PDGF
Antibodies	IgG
Coagulation cascade	Factor V, fibrinogen, tissue factor pathway inhibitor
Endothelial cell activation	TGF-β
Fibrinolysis	Plasminogen, PAI-1 and α_2-antiplasmin
Growth factors	PDGF, FGF, HGF, IGF-1 and EGF
Leucocyte recruitment	Chemokines: PF4, RANTES, β-thromboglobulin, ENA-78 and SDF-1α
Matrix breakdown	Hydrolytic enzymes MMP-2 and MMP-9
Membrane proteins	αIIbβ3, P-selectin, CD40L and TLT-1
Bacterial killing	Microbicidal proteins
Miscellaneous	Amyloid β-protein precursor and Gas6
Proteases	Protease nexin II
Platelet aggregation	Fibrinogen, fibronectin and VWF
Irreversible aggregation	Thrombospondin (locks fibrinogen bridges between αIIbβ3)

Major components of α-granules include clotting factors such as fibrinogen, VWF, factor (F) V, protein S and tissue factor pathway inhibitor (TFPI), the chemokines SDF-1α, PF4, β-thromboglobulin and CCL5 (also known as Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted [RANTES]), the growth factors platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) A and C. Platelet α-granules also express key transmembrane proteins including integrin αIIbβ3, P-selectin (CD62P), CD40L and TLT-1, the latter three only being expressed on the surface of activated cells.

Platelets are enriched in signalling and cytoskeletal proteins that enable them to undergo dramatic changes in morphology and which enable the aggregate to withstand the high shear forces of the vasculature. Platelets have a network of intracellular membranes known as the dense tubular system that release intracellular Ca^{2+} in response to the second messenger inositol 1, 4, 5-trisphosphate (IP3). They also have a network of invaginations of the surface membrane, known as the surface-connected canalicular system, which increase the surface area of the plasma membrane during platelet spreading.

Animal models in the study of platelet function

Genetically modified mice and other organisms, notably zebrafish, are used to address the *in vitro* and *in vivo* function of specific genes and proteins in platelets (and thrombocytes i.e. nucleated platelets – only mammals have anucleate platelets), but with the caveat of key differences to human platelets and vasculature with regard to rheology and protein composition. For example, the mouse genome lacks the gene for the only Fc receptor found on human platelets, FcγRIIA and mouse platelets do not express one of the major receptors for thrombin on human platelets, PAR1. There are also important differences in participation of protein isotypes in signalling cascades and various responses. The relevance of many of the *in vivo* models (such as tail bleeds and vessel wall injury by ferric chloride or laser ablation) to haemostasis and thrombosis in humans is also unclear, and no models fully recapitulate arterial thrombosis on ruptured atherosclerotic plaques. Nevertheless, the processes that govern platelet activation in mice and other species are shared with humans, and the value of animal models in analysing platelet activation and in studying haemostasis and thrombosis, and thereby establishing a foundation for ongoing and future clinical trials, is immense.

Platelet formation

Platelets are formed from megakaryocytes in the bone marrow. Approximately one million platelets are released into the blood every second. During megakaryopoiesis, the nucleus undergoes a process known as endomitosis that involves nuclear replication without cellular division, giving rise to DNA ploidy values that range from 4*n* to 128*n*, and cells that approach 50–100 μm in diameter (the largest of the bone marrow cells). The reason why endomitosis occurs is not fully understood, but it may simply reflect the need to increase the DNA content to enable the cell to expand its protein synthetic capacity to generate 2000–3000 platelets per megakaryocyte. In addition, it allows cell growth and differentiation to occur without interruption by nuclear and cell division.

The differentiation of bone marrow progenitor cells into megakaryocytes is regulated by the cytokine thrombopoietin (TPO). The TPO receptor, c-Mpl, is expressed on progenitor cells, megakaryocytes and platelets, and signals via the JAK family of tyrosine kinases. A mutation in *JAK2*, V617F, is the major cause of the myeloproliferative disorders, polycythemia vera, essential thrombocythemia and primary myelofibrosis (see Chapter 35). Platelet lifespan is determined by an internal molecular clock governed by time-dependent degradation of BCL-X_L which normally restrains BAK-1 and BAX from inducing apoptosis. In addition, platelets undergo desialylation as they age resulting in their eventual removal via the hepatic Ashwell-Morell receptor and induction of TPO synthesis. Circulating free levels of TPO are additionally controlled by binding and internalisation mediated through its receptor c-Mpl. The two feedback mechanisms provide a simple means of tightly controlling the platelet count. Thus, if the platelet count decreases, the free level of TPO rises and there is an increase in megakaryopoiesis and platelet formation. TPO mimetics (e.g. romiplostin and eltrombopag) are in clinical use for treatment of thrombocytopenia.

It has been proposed that megakaryocyte differentiation occurs in a defined compartment of the bone marrow known as the osteoblastic niche and that mature megakaryocytes subsequently migrate from this to sinusoidal endothelial cells in the vascular niche. Here, the megakaryocytes generate long thin processes known as proplatelets, which form platelets at their terminals. The proplatelet arms protrude between bone marrow sinusoidal endothelial cells and release immature preplatelets (which divide into platelets) and platelets into the bloodstream. As yet, it has not been possible to replicate the generation of *very large* numbers of platelets from a single megakaryocyte *in vitro*, possibly because of the interplay of the environment and role of shear. Several groups and biotech companies are active in this area which has the potential to replace platelet donation in transfusion medicine.

Thrombus formation

The events that underlie platelet adhesion and aggregation, and which lead to thrombus formation, at intermediate to high rates of shear (1000–5000/s) of the arteriolar system are discussed below and depicted in Figure 40.2.

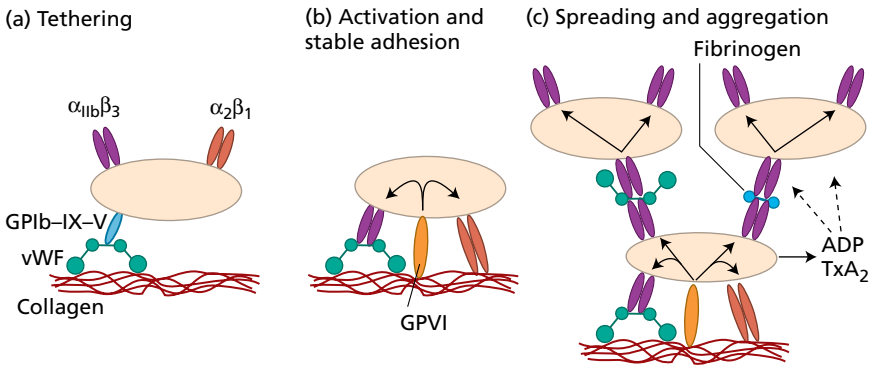

Figure 40.2 Thrombus formation at arteriolar rates of shear. Platelets are (a) tethered by VWF bound to immobilised collagen, which then (b) activates the low-affinity receptor GPVI leading to activation of integrins αIIbβ3 and α2β1, which bind to VWF/fibrinogen and collagen, respectively. This mediates stable adhesion and supports further activation of GPVI as a result of avidity. (c) The signals from GPVI and the two platelet integrins induce spreading of platelets on the matrix and release of the feedback messengers ADP and TxA_2 which activate further platelets. The generation of thrombin further supports platelet activation and generates fibrin-supporting clot retraction. The formation of a procoagulant surface supports formation of thrombin.

Platelet capture and stable adhesion

The initial event that takes place upon damage to the vasculature in the arteriolar circulation is the tethering or capture of platelets through the interaction of VWF with the GPIb-IX-V complex. VWF undergoes a conformational change on binding to collagen or in response to very high shear forces enabling it to bind to GPIbα. The fast on-rate of association between VWF and GPIbα enables capture of platelets at the high shear forces of the arteriolar system, but a fast off-rate of association means that this interaction is insufficient to give rise to stable adhesion. Thus, platelets translocate in the direction of flow on a surface of immobilised VWF (in the absence of collagen). Platelet capture is facilitated by the formation of membrane tethers, which are thin processes of membrane generated from the platelet surface by haemodynamic forces. These membrane tethers can extend up to 30 μm in length, with much of their length being derived from the large membrane component of the surface-connected canalicular system. The formation of membrane tethers helps to sustain adhesion within high-shear environments by minimising the drag on captured platelets.

The conversion to stable adhesion is dependent on activation of α2β1 and αIIbβ3 integrins, the collagen receptor GPVI, vessel-wall-generated thrombin and release of ADP. This transition can be readily demonstrated *in vitro* by comparing adhesion and aggregate formation when blood flows over immobilised VWF or a VWF/collagen surface, as illustrated in Figure 40.3. The majority of platelets translocate on a surface of VWF and form small stable aggregates only after several minutes. In comparison, platelets undergo rapid stable adhesion and form large aggregates on collagen. This process is mediated by the binding of plasma-derived VWF to collagen through its A3 domain, unmasking the binding site for GPIb within the VWF A1 domain and facilitating platelet capture and activation of GPVI by collagen, and release of the feedback mediators ADP and TxA_2.

The two major integrins that mediate stable adhesion on collagen are αIIbβ3 and α2β1 (GPIa/IIa), which bind to immobilised VWF and collagen, respectively. The role of α2β1 in this process is masked by the 50 times higher level of αIIbβ3, which is expressed at approximately 80,000 copies per platelet, with a further 40,000 copies on intracellular α-granules that become exposed on activation. The capture and recruitment of circulating platelets into a growing aggregate occurs in a similar way, with integrin activation mediated by the release of ADP and TxA_2, and the local formation of thrombin. Binding of VWF to integrin αIIbβ3 on activated platelets mediates tethering of non-activated platelets through GPIb-IX-V thereby recruiting them into the growing platelet plug.

At the lower shear rates (<500/s) in the venous system, platelets adhere directly to exposed subendothelial matrix proteins and undergo stable adhesion independent of VWF and GPIb-IX-V, with the release of ADP and TxA_2, and thrombin generation, sufficient to enable aggregate growth to occur.

Spreading

Platelets undergo a characteristic set of morphological changes on contact with a surface, known collectively as spreading, as illustrated in Figure 40.4. Initial shape change or rounding is followed by the generation of finger-like projections known as filopodia, which grow from the periphery of the cell, and the subsequent formation of lamellipodia. This is illustrated in Figure 40.4 with platelets spread on fibrinogen initially forming filopodia (100 s) with lamellipodia beginning to form at 200 s and being completely

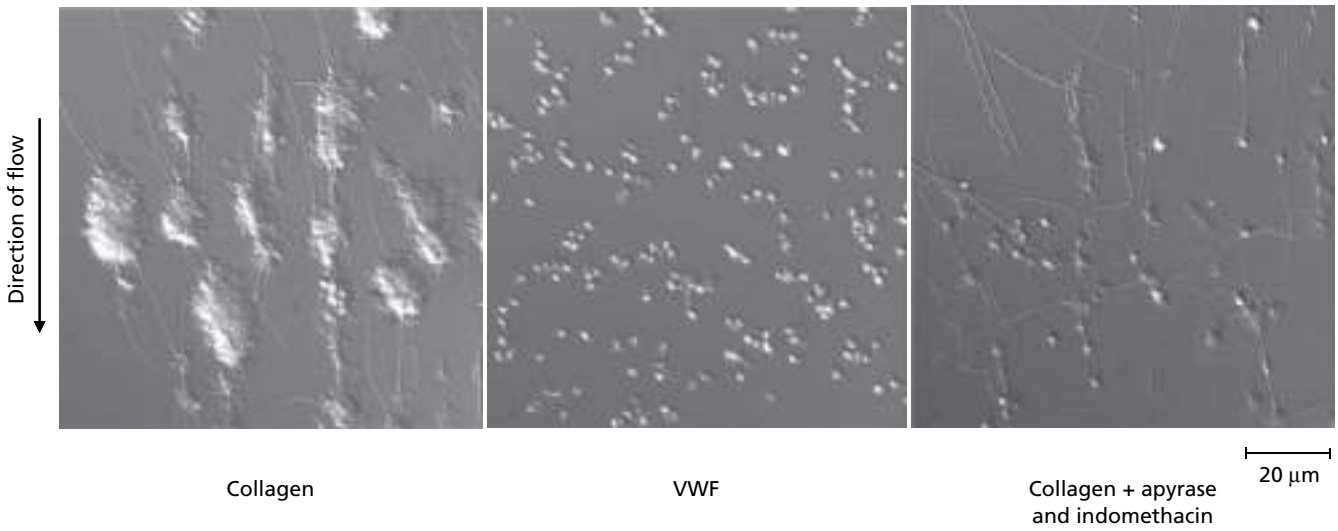

Figure 40.3 Platelet adhesion and aggregation at arteriolar shear. Human blood, anticoagulated with 40 µmol/L P-PACK, was flowed over collagen or VWF at a shear rate of 1000/s for 4 min and rinsed with Tyrode buffer for 5 min. Differential interference contrast images of adherent platelets were recorded. Where indicated, blood was pretreated with indomethacin (10 µmol/L) and apyrase (5 U/mL) to reduce the secondary mediators, TxA_2 and ADP respectively, prior to flow.

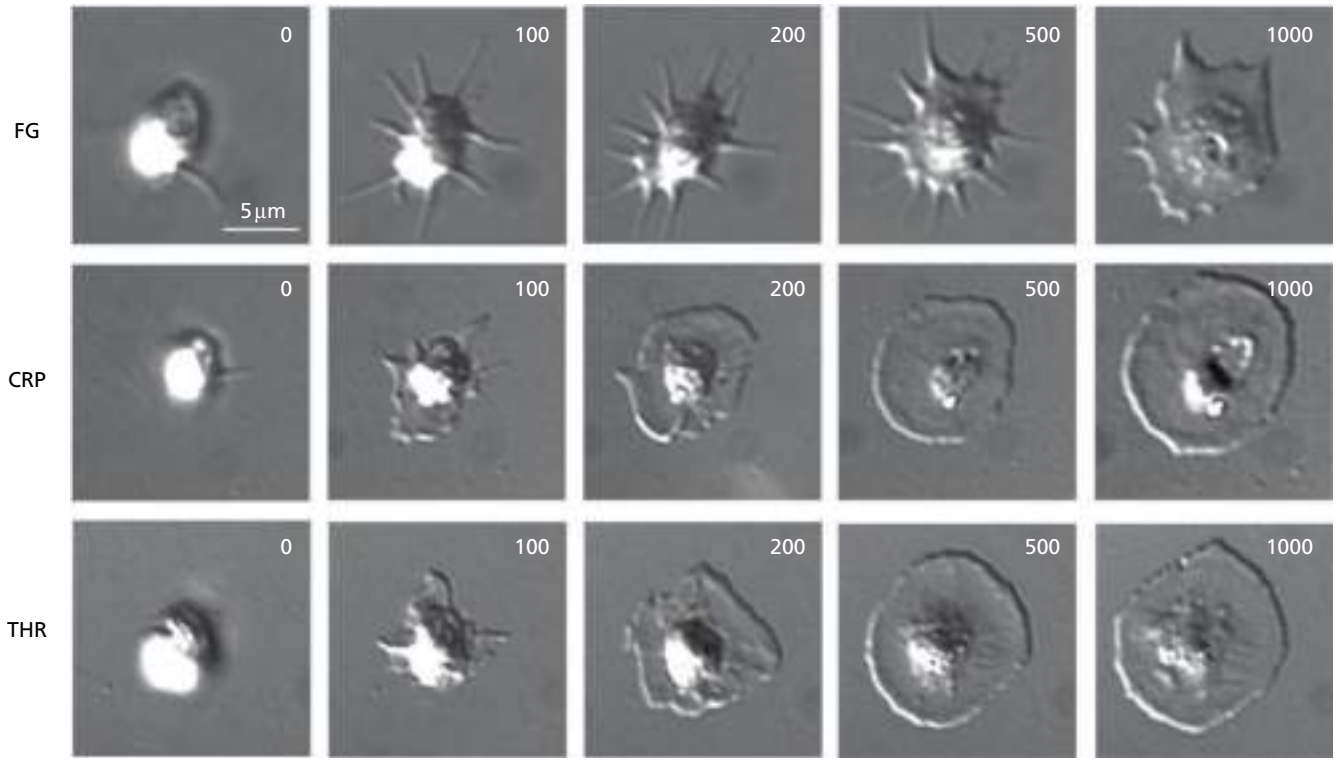

Figure 40.4 Morphological changes in washed human platelets followed adhesion. Human platelets were allowed to adhere to a surface coated with fibrinogen (FG), collagen-related peptide (CRP) or thrombin (THR). Representative morphology of a single platelet on each surface at specific time points (seconds) are shown (Source: Thornber *et al.* (2006) *FEBS. J.* **273**: 5032–43. Reproduced with permission of Wiley.)

formed at 1000 s. As these events proceed, granules and organelles are squeezed into the centre of the cell, resulting in a characteristic 'fried egg' appearance. These dramatic changes in morphology are brought about by a powerful severing and reassembly of the actin cytoskeleton. The spreading of platelets and formation of stress fibres helps to secure the platelet and thrombus against the flow and shear forces of the vascular system.

Granule secretion and TxA_2 formation

The secretion of ADP from dense granules and the *de novo* generation of TxA_2 from arachidonic acid, by the action of phospholipase (PL)A_2, play a feedback role in mediating platelet activation. Fusion of α-granules leads to expression of P-selectin on the platelet surface, which is the major ligand for P-selectin glycoprotein ligand (PSGL)-1 on leucocytes. The capture of leucocytes contributes to inflammatory events at the vessel wall including formation of neutrophil extracellular traps (NETs). Additional interactions further support the generation of NETs including the recent demonstration of binding of platelet αIIbβ3 to the choline transporter SLC44A2 on neutrophils.

Aggregation

Aggregation is the term used for the crosslinking of activated platelets through binding of bivalent or multivalent ligands to integrin αIIbβ3. It is distinct from agglutination which is a passive process mediated, for example, by the antibiotic ristocetin, which induces crosslinking of VWF to GPIb-IX-V. Ristocetin is routinely used to investigate possible defects or gain-of-function mutations in plasma VWF or GPIb.

Integrin αIIbβ3 is present in a low-affinity form on non-stimulated platelets and undergoes a conformational change to a high-affinity form in response to 'inside-out' signals. The subsequent binding of bivalent ligands to the integrin mediates platelet adhesion and aggregation. These interactions lead to clustering of αIIbβ3 on the platelet surface and the generation of 'outside-in' signals that regulate actin polymerisation (and therefore platelet spreading) and activation in synergy with other platelet receptors. Fibrinogen, VWF and fibronectin have similar affinities for integrin αIIbβ3, but fibrinogen is considered to be the major ligand because of its much higher concentration in plasma.

It has been proposed that aggregate growth is supported through homophilic and heterophilic interactions between additional platelet membrane proteins, either through the generation of intracellular signals or through adhesion. Examples include the role of semaphorin D in supporting signalling by GPVI and the binding of ephrins and eph-kinases which support activation through adhesion and signalling. In addition, an actin-dependent contractile mechanism has been shown to bring newly captured platelets into the aggregate. The overall significance of these interactions however is unclear and, as yet, there is no clear evidence of a bleeding diathesis resulting from defects in these 'late-events' in aggregation.

Fibrin generation and clot retraction

Critical to the formation of a stable aggregate, or thrombus, is fibrin, which is a polymerised fibrous protein generated by the action of the protease thrombin on fibrinogen. The formation of fibrin together with the platelets form the major components of the 'white' clot in the arterial circulation; this is in contrast to the 'red' clot in the venous system, which contains a high proportion of red blood cells. The true picture however is a spectrum from the arterial to the venous system, with a remarkable heterogeneity between vascular beds and individuals as elegantly reviewed by Weisel and Litvinov (2021).

The platelet thrombus is composed of core and shell regions

Advances in intravital microscopy and in fluorescent microscopy have revealed a much greater complexity in the composition of thrombi in mouse models than in *in vitro* assays. These studies have shown a temporal and spatial organisation of thrombi with a densely packed *inner core* composed of fibrin and an *outer shell* of loosely packed platelets held together by fibrinogen. The platelets in the core region are highly activated as shown by their dramatic change in morphology and the region is poorly penetrable to solutes (including fibrinolytics) whereas the shell is composed of large numbers of loosely packed platelets in their original biconcave shape. It has been proposed that the core reflects strong activation of platelets by thrombin (formed through the activation of tissue factor in the vessel wall) and the release of the feedback ligands ADP and TxA_2, as well as possibly activation by fibrin, which has been shown to activate GPVI. In contrast, thrombin is absent in the shell region and the levels of ADP and TxA_2 are lower (as they diffuse away from the shell) resulting in a much weaker degree of activation. In this model, the generation of ADP and TxA_2 are critical for the formation of the shell but not the core due to the redundancy with thrombin. This heterogeneity has significant implications for the mode of activation of antiplatelet and antifibrinolytic drugs.

The reader is referred to the review by Ivanciu and Stalker (2015) for further information on thrombus heterogeneity.

Procoagulant activity and microvesicle formation

Activated platelets provide a negatively charged phospholipid surface for the assembly of two multiprotein complexes that form part of the intrinsic and amplification pathways of coagulation, namely the tenase and prothrombinase complexes.

A complex of FIXa and FVIIIa on the negatively charged lipid surface converts FX to FXa (intrinsic tenase complex), which in turn forms a complex with FVa on the same surface to efficiently convert prothrombin to thrombin (prothrombinase complex). In this way, a large amount of thrombin is generated in the vicinity of the growing aggregate that serves to enhance platelet activation and convert fibrinogen to fibrin.

The platelet procoagulant response is dependent on exposure of phosphatidylserine (PS) elicited in response to stimulation by a combination of G protein-coupled and tyrosine kinase receptor agonists such as thrombin and collagen, respectively. PS exposure requires sustained entry of Ca^{2+} across the plasma membrane which can lead to zombification (or apoptosis) of platelets as a distinct phenotype with swelling, loss of granules and loss of aggregatory phenotype. This can be seen using fluorescent microscopy with distinct populations of platelets expressing PS (zomboid platelets) and activated αIIbβ3. A very small number of patients have been described with a mild bleeding disorder linked to defective procoagulant activity, a condition known as Scott syndrome. Scott syndrome is caused by mutations in the membrane transporter, transmembrane protein-16F (TMEM-16F).

The generation of procoagulant platelets shares similarities with the mechanisms that give rise to the formation of platelet extracellular vesicles (EVs), which are small membrane-bound vesicles of between 30 and 1000 nm. EVs and their functional significance is a highly active area that is held back by the challenge of studying such a small and heterogeneous population. EVs have been proposed to transfer microRNAs (miRNAs) to other cells to regulate gene expression as well as being biomarkers of platelet activation. Other biomarkers of platelet activation include soluble P-selectin and GPVI, with the latter being specific for platelet activation due to its restricted distribution.

Platelets in thrombo-inflammatory disease

For the first 100 years or more following their discovery in 1881, the focus of research on platelets has been on blood clotting. However, since the millennium, there has been a much wider appreciation of the role of platelets beyond haemostasis, notably in inflammatory processes and in vascular integrity. This has given rise to a new area of research known as thrombo-inflammation with the interplay of inflammation, platelets, coagulation factors and endothelial cells giving rise to plaques in the arterial system and thrombus formation on intact endothelial cell layers in the venous system (and conditions such as deep vein thrombosis). Thrombo-inflammatory processes are also observed in infectious diseases such as severe COVID-19 with endothelial cell activation being driven by the inflammation.

As described above, haemostasis in the arterial system is driven by exposure to the subendothelial matrix. This initiates a rapid cascade of events that lead to formation of a platelet plug in the subendothelium (haemostasis). Platelets are now recognised to participate in a second form of haemostasis in the venous system, known as inflammatory haemostasis, in which exposed sub-endothelial matrix promotes recruitment and activation of neutrophils and release of proteases that increase vascular permeability, leading to leakage of red blood cells. Unchecked by the adhesion of single and small clumps of platelets this generates characteristic red and purple spots on the skin known as petechiae. This is known as inflammatory haemostasis and is independent of integrin αIIbβ3 and GPIb-IX-V (Rayes *et al.* 2019). Petechiae are associated with low platelet counts and inflammation in the vessel wall. GPVI and a second receptor in platelets which signals through the same pathway, CLEC-2 (see below) have been shown to support inflammatory haemostasis in some but not all vascular beds in mouse models, but it is not known if this is the case in humans.

The pathological counterpart of inflammatory haemostasis is thrombo-inflammatory disease which is driven by a chain of inflammatory events that lead to endothelial cell activation, and recruitment of single or small clumps of platelets and coagulation factors leading to disorders such as deep vein thrombosis (DVT). We still know relatively little about this new area of thrombosis and the potential for therapeutic intervention, including by targeting GPVI and CLEC-2.

Platelet receptors

Receptors on platelets can be divided according to whether their major function is to mediate adhesion or to regulate activation (Figure 40.5).

Adhesion receptors

The major two adhesion receptors in platelets are GPIb-IX-V, which captures platelets in the high shear environment in the arterial circulation, and integrin αIIbβ3 which mediates stable adhesion and platelet aggregation. In addition, platelets express adhesion receptors for other matrix proteins including integrins α2β1, α5β1 and α6β1 which are receptors for collagen, fibronectin and laminin, respectively. Platelets also express receptors that mediate the interaction with other blood cells, including leucocytes, such as P-selectin which is expressed on activated platelets and binds to PSGL-1.

GPIb-IX-V

The GPIb-IX-V receptor consists of four subunits, GPIbα, GPIbβ, GPIX and GPV, and is restricted to platelets. There are 25,000 copies on the platelet surface making it the second most abundant membrane protein. It consists of two copies of GPIbα, GPIbβ and GPIX, and one copy of GPV. The principal ligand is

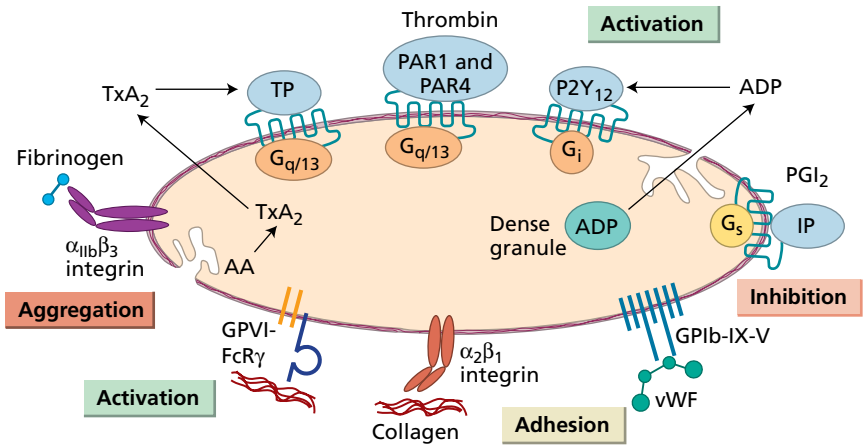

Figure 40.5 Schematic of major tyrosine kinase-linked and G protein-coupled receptors regulating platelets during haemostasis. The major signalling receptors that mediate platelet activation are the tyrosine kinase-linked receptor GPVI and the G protein-coupled receptors for TxA_2, thrombin and ADP. Of this group, the $P2Y_{12}$ ADP receptor is unique in that it synergises with the receptors for the other agonists to mediate powerful activation. The GPIb-IX-V complex is critical for platelet tethering and integrins α2β1 and αIIbβ3 for stable adhesion and, in the case of the latter, platelet aggregation. All three of these adhesion receptors also generate weak tyrosine kinase intracellular signals of uncertain significance. The prostacyclin receptor (IP) elevates cAMP and nitric oxide elevates cGMP (not shown) mediating powerful inhibition.

considered to be VWF, which binds to the GPIbα subunit. This also binds to other ligands as discussed below.

VWF consists of a series of multimers that vary in size from over 500 to over 10,000 kDa. The largest multimers of VWF are more effective in mediating platelet tethering and platelet activation. Ultra-large VWF is stored and released from endothelial cell Weibel–Palade bodies, and from activated platelets, and is broken down into the smaller multimers by the circulating metalloprotease ADAMTS-13. Deficiencies in ADAMTS-13 lead to the persistence of ultra-large VWF multimers in the circulation and a life-threatening thrombotic microangiopathy known as thrombotic thrombocytopenic purpura (TTP), characterised by VWF-rich platelet aggregates in the skin and vital organs (see Chapter 46). VWF circulates in plasma as a tightly bound complex with Factor VIII.

Upon binding to collagen, or upon exposure to ultra-high rates of shear, VWF undergoes a conformational change to an extended form that can reach several microns in length and which has multiple platelet and collagen binding sites. In this extended form, VWF is able to bind to the GPIb-IX-V complex and capture platelets in the high-shear environment of the arterial circulation. This is mediated by the fast on-rate of association of its A1 domain to the GPIbα subunit, but this also has a fast off-rate of association and signals from other receptors such as GPVI are required for integrin activation and stable adhesion.

GPIb-IX-V binds to several other cell-surface and plasma proteins, including integrin αMβ2 (also known as Mac-1 or CR3), P-selectin and thrombin (facilitating activation of PAR1 and PAR4 receptors). The interaction with αMβ2 and P-selectin contributes to attachment and transmigration of leucocytes through a mural thrombus, and the rolling of platelets on activated endothelium.

Mutations in *GPIbα*, *GPIbβ* and *GPIX*, but not in *GPV*, give rise to the severe bleeding disorder Bernard–Soulier syndrome (BSS), which is characterised by large platelets and thrombocytopenia (macrothrombocytopenia). Because of its severity, BSS is usually detected in early childhood, but in some cases, (notably in males) it may go undetected until adulthood.

Integrins

Integrins are heterodimeric-transmembrane proteins that mediate cell–cell and cell–matrix interactions and give rise to stable adhesive events and changes in cell morphology through the actin cytoskeleton. They are composed of α and β subunits which form a stable heterodimer. Intracellular signals, known as 'inside-out signals', convert integrins from a low to a high-affinity state promoting ligand binding. In turn, ligand binding generates 'outside-in signals' that remodel the cytoskeleton and generate other responses.

The integrin αIIbβ3 is the most abundant integrin on the platelet surface and constitutes approximately 15% of membrane proteins. Inside-out activation of αIIbβ3 is mediated by binding of the small G protein Rap1b to the cytoskeletal protein talin which in turn binds to the β3-tail. Outside-in signals from αIIbβ3 stimulate platelet spreading and clot retraction through PLCγ2 and the actin cytoskeleton, respectively. Activation of PLCγ2 by the integrin may be mediated through the platelet ITAM receptors GPVI and FcγRIIA (see below).

The so-called GPIIb-IIIa blockers were first introduced into the clinic in the 1990s. The original inhibitor, a chimeric Fab molecule, abciximab, was followed by two peptide inhibitors, eptifibatide and tirofiban, which bind to the Arg-Gly-Asp (RGD) binding sequence in the pocket of αIIbβ3. The RGD sequence is a common binding motif in matrix proteins including fibronectin, VWF and fibrinogen, although interestingly the latter binds to integrin αIIbβ3 through a KQAGDV sequence at the C-termini of its two γ-chains. GPIIb-IIIa blockers are used in the treatment of unstable angina and in percutaneous coronary interventions with their use limited to a hospital setting because of the risk of bleeding. Trials with orally active GPIIb-IIIa blockers were stopped due to increased mortality.

Four other integrins are found on platelets, these are: the collagen receptor α2β1, the fibronectin receptor α6β1, the laminin receptor α6β1 and the vitronectin receptor αvβ3. All of which are also found on other cells. Integrin α2β1 is considered to be the most important of these in platelet function as the result of binding to collagen. Their roles, however, are masked by integrin αIIbβ3, due to its much higher level of expression. No patients with a platelet-based bleeding disorder caused by variants in any of these four integrins have been identified. Conversely, mutations in either subunit of αIIbβ3 give rise to a major bleeding diathesis, known as Glanzmann thrombasthenia which is usually detected in early childhood due to its severity. Several hundred mutations have been identified with the majority leading to reduced expression enabling diagnosis by flow cytometry.

Platelet signalling receptors

Platelets are regulated by two major classes of signalling receptors, namely G protein-coupled receptors (GPCRs) and tyrosine kinase-linked receptors. Despite differences in the nature of their agonists and mechanisms of activation they stimulate common pathways, and cross-talk between the two receptor classes is a key feature of platelet activation. As an example, both G protein-coupled and tyrosine kinase receptors stimulate isoforms of phospholipase (PLC), namely β and γ, respectively, that generate the second messengers, IP3 and 1, 2-diacylgycerol (DG). They stimulate distinct isoforms of phosphatidylinositol 3-kinase (PI 3-kinase) which generates the lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate (PIP3), which recruits proteins with a pleckstrin homology (PH) to the membrane including PLC and the tyrosine kinase Btk. In addition, G protein-coupled receptors stimulate Rho family small G proteins and adenylyl cyclase, mediating cytoskeletal rearrangement and platelet inhibition, respectively.

There are four classes of heterotrimeric G proteins which take their name from the α subunit which binds to the β and γ subunits. Three of the four classes of α subunit are stimulatory, with G_q activating PLC β (PLCβ), G_{13} activating Rho family small G proteins, and G_i activating PI 3-kinase and inhibiting adenylyl cyclase. G_s mediates inhibition through activation of adenylyl cyclase. Thrombin and TxA_2 receptors stimulate G_q and G_{13} family G proteins, and ADP $P2Y_{12}$ receptor stimulates G_i. Prostacyclin and adenosine receptors stimulate G_s.

The most powerful of the activating tyrosine kinase receptors signal through a motif that is found in Fc and antigen receptors in other blood cells known as an immunoreceptor tyrosine-based activation motif (ITAM) and composed of two YxxL sequences (single amino acid code) separated by 6–12 amino acids. Platelets express three ITAM receptors, GPVI-FcR γ-chain complex, the low affinity immune receptor FcγRIIA and the C-type lectin-like receptor CLEC-2. One ITAM sequence is present in each of the FcR γ-chain subunits and in the cytosolic tail of FcγRIIA. The conserved tyrosines are phosphorylated by constitutively active Src family kinases in the membrane enabling binding of the tandem SH2 domains in the tyrosine kinase Syk. This leads to the autophosphorylation of Syk and a signalling pathway that involves a third family of tyrosine kinases, the Tec family, with Btk being the major isoform in platelets, which culminates in the activation of PLCγ2. CLEC-2 has a single YxxL group in its cytosolic tail, known as a hemITAM, with activation of Syk crosslinking two receptors. A schematic of ITAM signalling is shown in Figure 40.6.

ITAM receptors are inhibited by a class of membrane protein that contain a closely related motif known as an immunoreceptor tyrosine-based inhibition motif (ITIM), composed of two repeats of a YxxI/V/L sequence. Phosphorylation of the two conserved tyrosines in the motif leads to recruitment of the two tyrosine phosphatases to the membrane, SHP-1 and SHP-2, which dephosphorylate key proteins in the ITAM pathway. G6b-B is the only ITIM to be shown to have a major role in regulating platelet activation by ITAM receptors.

G protein-coupled receptors

$P2Y_1$ and $P2Y_{12}$ ADP receptors

ADP is a critical feedback agonist mediating activation through the synergy of $P2Y_1$ and $P2Y_{12}$ receptors, which activate G_q and G_i family heterotrimeric G proteins, respectively. The physiological significance of the $P2Y_1$ receptor is uncertain due to its low level of expression (~150 copies per platelets) and redundancy in its mode of signalling with the thromboxane TP receptor and the two thrombin PAR1 and PAR4 receptors. $P2Y_{12}$ is the target of the widely used antiplatelets, the thienopyridines clopidogrel and prasugrel and the ADP analogue ticagrelor. The $P2Y_{12}$ receptor signals through the G_i of G proteins and undergoes powerful synergy with G protein-coupled and tyrosine kinase-linked receptors that activate PLC isoforms even though it only induces weak activation on its own. Over 50 patients have been reported with function-disrupting mutations in $P2Y_{12}$ in association

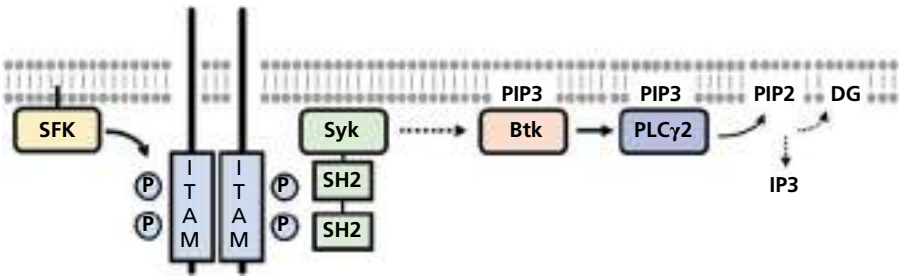

Figure 40.6 Schematic of ITAM signalling. An ITAM has the conserved sequence $YxxLx_{6-12}YxxL$ (single amino acid code). Receptor crosslinking induces phosphorylation of the two conserved tyrosines by a Src family kinase (SFK) leading to binding of the tyrosine kinase Syk through its tandem SH2 domains. Syk regulates a signalling cascade (depicted by the dotted line) consisting of adapter protein and SFKs that leads to activation of the Tec family kinase, Btk, and PLCγ2, and the generation of the second messengers inositol 1, 4, 5-trisphosphate (IP3) and 1, 2-diacylglycerol (DG). Platelets express two ITAM receptors, GPVI-FcR γ-chain and FcγRIIA. Platelets also express the hemITAM receptor CLEC-2 which has a single YxxL sequence in its cytosolic tail with Syk crosslinking two phosphorylated receptors. P depicts phosphorylation of the ITAM sequences: other phosphorylations are not shown.

with a bleeding diathesis. As yet, no patients have been identified with mutations in $P2Y_1$.

There are significant differences between the two thienopyridine $P2Y_{12}$ receptor antagonists, clopidogrel and prasugrel, and ticagrelor. Clopidogrel is a prodrug that is metabolised by the liver to an active metabolite that covalently modifies, and therefore irreversibly inhibits, $P2Y_{12}$. The related thienopyridine prasugrel is metabolised to the same active metabolite but through a pathway that is not governed by polymorphisms in cytochrome P450 enzymes. Ticagrelor is an orally available non-competitive antagonist that also inhibits uptake of adenosine, possibly accounting for its more powerful action, which needs to be balanced against an increased risk of bleeding.

Thromboxane TP receptor

Ca^{2+}-mobilising receptors activate PLA_2 and induce the liberation of arachidonic acid in platelets for metabolism by lipoxygenase and cyclooxygenase enzymes. The latter leads to further metabolism by thromboxane synthase and the generation of TxA_2 which activates platelets through the thromboxane prostanoid (TP) receptor. TxA_2 is rapidly metabolised to the stable metabolite TxB_2 which is used as a measurement of the activation of the cyclooxygenase pathway. The stable mimetic, U46619, is used in experimental and diagnostic studies due to the instability of TxA_2.

The antiplatelet action of aspirin is mediated through covalent modification of platelet cyclooxygenase and inhibition of TxA_2 formation. The irreversible nature of the acetylation, in combination with the inability of platelets to synthesise significant levels of proteins, means that the concentration of aspirin required for inhibition is lower than that required to target cyclooxygenase in other cells, and is the rational for use of 'low dose' aspirin as an antiplatelet agent. Aspirin has been shown to reduce the mortality of myocardial infarction and major vascular events by approximately one-quarter.

A handful of patients have been shown to have function-disrupting mutations in the thromboxane receptor, many of which are heterozygous, and an even smaller number have shown to have homozygous mutations in $cPLA_2$. The low number of patients could reflect redundancy in the thromboxane pathway in platelet activation or increased embryonic lethality.

PAR1 and PAR4 thrombin receptors

Thrombin is the most powerful of the platelet agonists inducing activation through the proteolytic cleavage of PAR1 and PAR4 receptors, thereby generating tethered ligands that induce receptor activation. The action of the two thrombin receptors is terminated by internalisation. Synthetic peptides corresponding to the newly exposed receptor-specific sequences known as thrombin receptor-activating peptides (or TRAPs) are used to selectively activate the two PAR receptors. It is proposed that PAR1 mediates rapid activation and that PAR4 mediates sustained activation, although this is an over-simplification. PAR1 and PAR4 signal through G_q and G_{13} heterotrimeric G proteins activating PLC and Rho family G proteins. An orally active PAR1 receptor antagonist voraxapar was approved by the FDA as an antiplatelet agent in 2014 for patients with a history of myocardial infarction or peripheral arterial disease but has not gained widespread use due to cost and risk of bleeding. A PAR4 antagonist, BMS-986120, has been developed and was entered into a phase III trial which was prematurely stopped for reasons that are not known. No

patients with defects in either receptor have been identified, possibly because the absence of one receptor is masked by the other or because homozygous mutations are lethal due to their role in other cell types, including vascular endothelial cells.

5-HT$_{2A}$ receptors

Platelets contain very high levels of 5-HT taken up by an active transport mechanism and stored in dense granules. Release of 5-HT induces powerful vasoconstriction, thereby limiting blood loss. 5-HT mediates very weak platelet activation and this plays little or no role in haemostasis or thrombosis.

α_{2A}-adrenoceptor

Adrenaline activates the α_{2A}-adrenoceptor, which is coupled to the G_i family of proteins, predominantly G_z. As is the case for the P2Y$_{12}$ ADP receptor, the α_{2A}-adrenoceptor undergoes synergy with Ca^{2+}-mobilising receptors but has little effect on its own. While studies in α_{2A}-adrenoceptor knockout mice have provided evidence for a minor role in supporting haemostasis, there is little evidence to suggest this is the case in human. Adrenaline is frequently used in aggregation studies on patients with a suspected platelet-based bleeding disorder, although interpretation of results is challenging as a proportion of healthy donors are unresponsive. As such, it is difficult to see the rational for the continued use of adrenaline.

Tyrosine kinase receptors

GPVI-FcRγ

Collagen is the most thrombogenic component of the subendothelial matrix with up to nine forms in the vessel wall, with types I and III being the predominant two in the deeper layers and type IV in more superficial layers. The major activating receptor for collagen on platelets is GPVI which is expressed at ~4000 copies per platelet. The majority of collagens activate GPVI, with the sheet-like collagen type IV being a notable exception.

Collagen is composed of three helical chains, which form a superhelical structure that is interrupted by non-helical regions. Synthetic collagen peptides based on repeating trimers in the helix have been designed to bind selectively to GPVI or to the collagen integrin receptor α2β1. Peptides that are specific to GPVI have a backbone of glycine-proline-hydroxyproline (GPO) residues and are known as collagen-related-peptides (CRP), whereas peptides that bind to α2β1 are interspersed with one or more GER-containing motifs, such as GFOGER.

GPVI is also activated by a large number of endogenous and exogenous ligands, including fibrin and the snake toxin convulxin, and has been proposed to be a pattern recognition receptor to account for this promiscuity. A key feature in GPVI-activating ligands is the presence of multiple epitopes leading to receptor clustering. The potential significance of the activation of GPVI by so many ligands, including fibrin, is unclear.

GPVI is found only in platelets and megakaryocytes. It has been shown to be absent in two patient groups. The first to be identified were individuals with immune thrombocytopenia caused by auto-antibodies to GPVI which induce shedding of the glycoprotein receptor and a lowering of the platelet count. These patients have a mild to severe bleeding diathesis depending on the degree of thrombocytopenia. The second consists of a small number of patients with an inherited defect that results in the absence of *GPVI* expression. The majority of these have the same mutation that introduces a stop codon prior to the membrane sequence and prevents expression. The patients have a mild bleeding diathesis that diminishes with age. To date, eleven unrelated families have been identified in Chile with this mutation but as yet none have been found outside of the country. The insertion is believed to be present in just under 0.3% of the Chilean population indicating that there are ~4000 GPVI-deficient individuals in the country. Identifying many more of these will help to establish whether a bleeding diathesis is always present in the absence of GPVI and whether loss of the collagen receptor provides protection from cardiovascular disease. This has important implications for development of GPVI inhibitors as novel antiplatelets.

A GPVI blocking Fab, glenzocimab (ACT-017), has entered a phase II trial for ischaemic stroke in addition to the best emergency standard of care. The results of this study are eagerly awaited. Small molecule inhibitors of Syk and Btk tyrosine kinases used in the treatment of immune thrombocytopenia and B cell malignancies, respectively, also block GPVI and are well tolerated. The advantage of inhibitors of Syk and Btk is their oral availability and more powerful action due to inhibition of other tyrosine kinase signalling receptors. On the other hand, they will also have an increased spectrum of off-target effects. As yet, no inhibitor of Syk or Btk has entered into trial as an anti-thrombotic.

CLEC-2

CLEC-2 is a C-type lectin-like receptor that is expressed at high level on platelets and at a lower level on a subset of myeloid cells. The endogenous ligand for CLEC-2 is the transmembrane protein podoplanin, which is found in many cell types including epithelial cells, inflammatory macrophages, stromal cells and lymphatic endothelial cells. Podoplanin is absent in the vasculature consistent with a negligible role for CLEC-2 in haemostasis.

Mice with a constitutive deletion of podoplanin or CLEC-2 exhibit blood-lymphatic mixing and impaired development of the cerebrovasculature, and die shortly after birth due to a failure in lung inflation (as a result of fluid build-up in the lymphatics). The deletion of CLEC-2 post-development is well tolerated, and

the primary role of the podoplanin-CLEC-2 axis in the adult appears to be in supporting inflammation following upregulation of podoplanin on macrophages and stromal cells. CLEC-2 has also been shown to support inflammatory haemostasis in combination with GPVI in mice models.

The podoplanin-CLEC-2 axis has been shown to drive thrombo-inflammation at sites of inflammation in the vessel wall following stasis or infection in mouse models. Podoplanin is also upregulated in human in the inflamed liver and in DVT suggesting it may represent a novel target for intervention. CLEC-2 is also activated by haem suggesting it may play a role in haemolytic microangiopathies, including haemolytic urinary syndrome and sickle cell disease.

FcγRIIA

The human but not rodent genome encodes for a low-affinity immune receptor, FcγRIIA, which is expressed on a variety of leucocytes and on platelets, and binds to the Fc tail of IgG presented as immune complexes. FcγRIIA is expressed at a similar copy number to GPVI and CLEC-2.

Activation of FcγRIIA underlies heparin-induced thrombocytopenia (HIT) which is caused by antibodies to PF4 in complex with heparin. In a small number of individuals, the presence of these can give rise to life-threatening thrombosis. HIT is thought to be mediated by preformed antibodies to PF4 that form part of an evolutionary defence mechanism against infection. This explains the unusually rapid time of onset within 5–30 days of first exposure to heparin.

Recently, a very rare (~1 in 50,000) disorder with a high rate of mortality (20–50%), known as vaccine-induced-immune-thrombotic-thrombocytopenia (VITT) has been recognised that has a number of similarties to HIT. VITT has an onset within 5–30 days following the first dose of adenovirus-based vaccines against COVID-19 and is associated with high levels of D-dimer and PF4-antibodies despite the patients not having been exposed to heparin. The binding of PF4 to the adenovirus has been proposed to trigger the generation of the high titre of high-affinity antibodies to the chemokine which leads to the formation of circulating immune complexes and activation of FcγRIIA on platelets and neutrophils. It is unclear why only 10% of recipients form antibodies to PF4 and why only a fraction of these go on to have VITT.

A similar mechanism to HIT and VITT has been speculated in other rare disorders associated with catastrophic thrombosis.

Inhibitory pathways

Cyclic nucleotides

Platelets have powerful inhibitory mechanisms that ordinarily prevent activation in intact healthy vessels. The major inhibitory pathway is through elevation of the cyclic nucleotides cGMP and cAMP which mediate their effects by activation of cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA). The two kinases phosphorylate a broad range of shared and distinct substrates resulting in the inhibition of small G proteins, Ca^{2+} release, cytoskeletal remodelling and PLC. Cyclic nucleotides are metabolised by a family of phosphodiesterases (PDEs), including PDE3 which is a substrate for PKG leading to elevation of cAMP. Thus, some of the effects of cGMP are mediated by an increase in cAMP. Cilostazol and dipyridamole selectively inhibit PDE3 and PDE5 isoforms and are used as weak antiplatelet agents, often in combination with aspirin. One of the prominent substrates for both PKA and PKG is vasodilator-stimulated phosphoprotein (VASP), which is used to monitor activation by flow cytometry.

The major pathway for regulation of cGMP is through release of NO from endothelial cells which is also mimicked by nitrate-based vasodilators. Platelets express several G protein-coupled receptors that couple to the G_s heterotrimeric G protein, including the prostanoid EP and IP receptors, and the adenosine A1 receptor. Prostacyclin released from endothelial cells is considered to be the major regulator of cAMP in platelets.

ITIM receptors

Platelets express several ITIM-containing membrane proteins including PECAM-1 and G6b-B, and TLT-1 which is present on α-granules. PECAM-1 was the first membrane ITIM protein to be identified but its role remains uncertain as mouse models have shown only a mild phenotype and patients with defects in PECAM-1 have not been reported. In contrast, studies on mouse models and a handful of individuals who lack G6b-B have revealed a critical role in maintaining the levels of GPVI and CLEC-2 and the platelet count. This is presumed to be mediated by inhibition of constitutive signalling by the two receptors which lead to their shedding from the membrane and platelet removal by the reticuloendothelial system. The cytosolic tail of G6b-B is constitutively phosphorylated on tyrosines in its ITIM which recruit the tyrosine phosphatases SHP1 and SHP2 to the membrane. The functional significance of TLT-1 on platelet α-granules is not known.

Platelet-based bleeding problems

Bleeding can be due to abnormalities in platelet function or number, or due to antiplatelet medication. Patients with platelet disorders present with a typical mucocutaneous bleeding pattern of variable severity and are often prone to bleeding during surgery and trauma. However, inherited platelet defects are an uncommon cause of bleeding and are difficult to diagnose and manage, with wide variation in practices between laboratories. Before any platelet function test is requested, a full clinical

history is taken to help determine the underlying cause of the bleeding problem. This includes studying the pattern of bleeding, whether it is lifelong or recent, triggered by trauma (e.g. surgery or accident), is present within other family members and associated with any medication the patient may be taking. Von Willebrand disease (VWD) is the most commonly inherited bleeding disorder but presents with a platelet-like bleeding pattern and so measurement of VWF and other coagulation factors is performed ahead of platelet function assays. Although bleeding histories are subjective and can vary within a lifetime, they remain an important screening method for diagnosing a potential platelet defect. The International Society of Thrombosis and Haemostasis (ISTH) has introduced a bleeding assessment tool (BAT) based on a questionnaire which is now routinely used. However, it is also important to exclude acquired defects of platelet function, including those caused by concomitant prescribed and non-prescribed medications and other clinical disorders. A complete drug history is therefore important, as aspirin and non-steroidal anti-inflammatory drugs are the commonest causes of an acquired platelet defect and so testing may have to be deferred and repeated.

Inherited platelet defects can be classified according to the severity of bleeding, with severe disorders such as Glanzmann thrombasthenia and BSS usually being detected very early in life, while more mild bleeding disorders (e.g. due to $P2Y_{12}$ deficiency) often not being detected for many years and influenced by other acquired and genetic defects. There is overlap between symptoms in milder platelet defects and the normal population and it is now widely recognised that most platelet-based bleeding defects are polygenic, with even the severity of Glanzmann thrombasthaenia and BSS varying markedly between individuals. Further information on inherited platelet bleeding disorders is given in Chapter 44.

Platelet function testing

Platelet function tests are primarily used to aid diagnosis of patients presenting with bleeding problems. Normal platelet function is highly dependent on extracellular Ca^{2+} and Mg^{2+} concentrations and so the choice of anticoagulant is important. Most current testing is still performed on citrated blood within a few hours of sampling.

Global tests of platelet function are initially used as screening tests during the laboratory investigation of individuals with suspected haemostatic defects. Since global tests do not enable specific diagnosis of platelet disorders, they are normally performed as the first part of a two-step strategy that requires further testing with more specialised assays to confirm or refute any clinical diagnosis. The most commonly proposed rationale for testing global platelet function as a first-line investigation is exclusion of a platelet function disorder so that further specialised testing can be avoided. For this reason, global platelet function tests are usually initially performed at the same time as global assays of coagulation pathway function (prothrombin time activated partial thromboplastin time and functional fibrinogen assay), VWF screening tests (VWF:Ag, VWF:RCo and FVIII:C) and measurement of platelet number with a full blood counter. The most widely performed test for initial global screening platelet function disorders are currently the closure time within the platelet function analyser (PFA)-100/200 with the bleeding time no longer recommended.

The bleeding time was the first *in vivo* test of platelet function and is performed by timing the arrest of bleeding from standard-sized cuts made in the skin of the forearm. Most clinicians consider the test to be poorly reproducible, invasive, insensitive (particularly to mild platelet defects) and time-consuming. Despite these drawbacks, the test is surprisingly still used in some countries. A number of *in vitro* tests that attempt to accurately simulate platelet function have been developed. The most widely used of these is the PFA-100/200. This simulates high-shear platelet adhesion to foreign surfaces (e.g. collagen) and monitors the drop in flow rate as a small aperture is closed by platelet adhesion and aggregation. The PFA-100/200 instrument provides an optional replacement of the bleeding time as a limited screening test. Although the instrument has a good negative predictive value for eliminating *severe* platelet defects and VWD, it is very important that further diagnostic tests are still performed if clinical suspicion of a platelet defect is strong.

Automated cell counting of whole blood in the modern full blood count investigation is an essential screening test in patients with abnormal bleeding. Modern blood counters can rapidly detect abnormalities in platelet number, platelet size distribution and mean platelet volume (MPV) and provide a reliable method for screening samples from patients, provided good quality control procedures are followed within the laboratory. Normal results will therefore rapidly eliminate thrombocytopenia and anaemia as potential causes of bleeding and ensure that any platelet function tests that are being performed will not be affected by low platelet counts. If any abnormalities in platelet count, MPV or distribution are flagged by the instrument, then a blood smear should be examined to confirm defects in platelet number, size and granule content. Whole-blood morphology may also assist in the diagnosis of platelet disorders by indicating abnormalities such as red cell schistocytes in TTP or neutrophil inclusions and giant platelets in MYH-9-related disorders.

Depending on the results of clinical and laboratory screening of patients, a series of diagnostic platelet function tests are then performed. Since the 1960s, light transmission aggregometry (LTA) became the gold standard of platelet function testing and has revolutionised our ability to identify and diagnose a wide variety of platelet defects. Conventional Born aggregometers monitor the changes in light transmission that occur in a suspension of platelets in plasma or a physiological buffer that are stimulated with different concentrations of various agonists (e.g. ADP, collagen, adrenaline and ristocetin). Typical parameters

recorded from LTA tracings include shape change, lag phase, rate of aggregation, and maximal and final extent of aggregation. These parameters, coupled with the pattern of primary and secondary aggregation responses obtained with different dosages of different agonists, enable the experienced operator to diagnose specific signalling defects. Modern aggregometers are now multichannel, fully computerised, easy-to-use compact instruments and some can also simultaneously measure ATP secretion levels by luminescence. About 96 well plate aggregation testing is also available but is primarily used for research purposes and is not yet in routine use in the clinical testing laboratory.

Measurement of the storage and release of the dense granular nucleotides can also be performed by a variety of methods for confirming either storage and/or release defects, although recent surveys suggest that measurement of secretion is underused. The interpretation of aggregation traces remains challenging due to the feedback actions of ADP and TxA_2. Whole-blood impedance-based aggregometers are now common and measure the changes in electrical resistance between two electrodes caused by platelet adhesion and aggregation after addition of agonists, but are heavily influenced by platelet count.

Flow cytometry provides an exquisite, sensitive and powerful tool for studying and diagnosing various platelet defects. Flow cytometric analysis of platelets is performed in fresh whole blood or in platelet-rich plasma, and the technique can be used with very small fluid volumes, even in thrombocytopenia. This technique is used to determine the copy density of platelet membrane glycoproteins and receptors and is therefore useful for confirming the absence of various glycoproteins or receptors in disease.

Platelet function testing can also be performed and the ability of platelets to degranulate, express activation markers (e.g. P-selectin) and expose procoagulant phospholipids can be studied. A major limitation of the above tests is that they are performed at low shear conditions and therefore do not mimic accurately many of the important physiological processes of platelet adhesion, activation and aggregation that occur at higher shear rates *in vivo*. There is clearly still no gold standard for platelet testing, and indeed there may never be, and LTA remains the most widely used test. However, a variety of screening and diagnostic tests coupled with an accurate bleeding history will always be required to confirm a platelet-based bleeding disorder, simply because of the heterogeneity in the range of disorders encountered coupled with the cross-talk between receptors.

Platelets and thrombosis

There has been widespread interest in using platelet function tests to reliably detect platelet hyper-reactivity in patients with arterial thrombosis but this is not recommended in part because these are complex diseases resulting from insults to the vessel wall. Nevertheless, this interest, as well as clinical discussion of aspirin or clopidogrel 'resistance', which is more correctly termed as treatment failure, has resulted in the development of a variety of instruments for platelet function testing (e.g. Platelet Mapping System, VerifyNow, Plateletworks and Aspirinworks). The VerifyNow test was originally developed as a whole-blood cartridge-based test to monitor αIIbβ3 blockade because of the narrow therapeutic window and increased bleeding risk associated with these types of drugs. The test has also been adapted to measure aspirin and $P2Y_{12}$ blockade with two other cartridge formulations.

Platelet responsiveness to antiplatelet drugs can be easily monitored by the tests mentioned, as well as by LTA, whole-blood aggregometry and PFA-100/200. Poor responders or 'resistant' individuals can therefore be identified, although it is still unclear how to manage them and whether there is a clinically relevant relationship between poor response and outcome. Emerging evidence suggests that the true incidence of so-called aspirin resistance is probably very low (when non-compliance is accounted for) and that many studies are measuring platelet hyper-reactivity and not blockade of cyclooxygenase type 1. Although recent meta-analyses suggest that this could nevertheless be clinically informative, it is still unclear how to reliably identify these patients, with which test and cut-off (as different tests give different results) and then actually how to manage these patients.

Testing is therefore still very much in the clinical research setting. Indeed, ISTH Scientific and Standardisation Committee guidelines suggest that aspirin resistance should not be routinely monitored and the wider question remains whether routine testing of platelet hyper-reactivity and/or drug responsiveness is actually clinically necessary. There is a spectrum of platelet responsiveness within the normal population and those individuals with a stable or even an acquired hyper-reactive phenotype could theoretically be at higher risk of thrombosis.

Clopidogrel resistance is a different phenomenon as this is a prodrug that requires metabolism by liver cytochrome P450. This results in the generation of an active metabolite that irreversibly inhibits the $P2Y_{12}$ receptor. Differences in the efficiency of metabolism between individuals (related to their P450 genotype) taking standard clopidogrel dosing results in a spectrum of responsiveness but results from recent meta-analyses suggest that the overall significance of this remains unclear and do not recommend patients for genotyping. While there is a variety of tests available to monitor the efficacy of clopidogrel, including LTA, whole-blood aggregation (Multiplate), VASP phosphorylation and VerifyNow, one of the problems facing the investigator is not only which test to use, but also the definition of a suitable cut-off defining a non-response and how to effectively manage these individuals. Increased use of ticagrelor has also reduced any need for testing. Although there are emerging clinical cardiology guidelines, they do not include routine monitoring of platelet responsiveness because of the wide spectrum of response between individuals and lack of strong evidence of the relationship between testing and clinical outcome.

Genetics of platelet function disorders

Inherited platelet function disorders (IPFDs), associated with normal or reduced platelet counts, are individually rare, but collectively they account for a significant proportion of bleeding diatheses. Inherited platelet disorders include defects in adhesion, receptor signalling, secretion, cytoskeleton, procoagulant activity and production.

Identification of the underlying genetic defects is difficult and often complex in the majority of PFD cases. This is due to the variable clinical expression of the bleeding symptoms and the relative redundancy of known platelet receptor and signalling pathways. Causative mutations for PFDs have therefore only been identified in a small number of patients. For example, the number of patients with mutations in the G_i-coupled $P2Y_{12}$ ADP receptor is still relatively low despite its critical role in platelet activation. In most cases, the defect is frequently recessively inherited and the patients are either homozygous or compound heterozygous for *P2RY12* mutations. Similarly, only a handful of functionally disrupting mutations have been reported in the G_q-coupled thromboxane receptor, of which all patients are heterozygous for the mutation. A significant number of mutations in PFDs are indeed heterozygous, but many are unlikely to cause extensive bleeding in isolation. This is illustrated in some patients with heterozygous *P2RY12* defects diagnosed with type 1 VWD, a finding which emphasises the heterogeneity and polygenic nature of PFDs and reflects the complexity of disorders of primary haemostasis.

In the last 25 years, DNA-based technologies have played a significant role in the characterisation and diagnosis of PFDs. Currently, the most time- and cost-efficient means of screening genes is whole exome sequencing (WES). WES enables the detection of genetic variation throughout the protein-coding fraction of the genome. The main challenge in applying WES for gene discovery is filtering out the large numbers of irrelevant variants (typically in excess of 20,000 per individual). However, the 'unbiased' nature of this approach means we are not limited by preconceptions as to likely causative mutations. This powerful technique is illustrated in regard to platelet biology by the discovery of the gene, neurobeachin-like 2 (*NBEAL2*), for Grey platelet syndrome, an autosomal recessive bleeding disorder characterised by large platelets that lack α-granules. WES has also been utilised for the identification of activating causative mutations in *STIM1* and *ORAI1* for Stormorken syndrome leading to increased platelet procoagulant activation. A more extensive section on congenital platelet disorders is covered in Chapter 44.

The most recent sequencing technology to emerge is whole genome sequencing (WGS), which is now the method of choice as costs are reduced and sequence variation databases are improved. Alternatively, a more gene-specific targeted platform may be the method of choice to reduce the time to diagnosis, by allowing the capture and sequencing of a panel of genes of relevance to bleeding and thrombotic disorders, and is playing an increasingly important role in the first-line investigation of patients with PFDs. Insights into the underlying mechanisms of platelet function through genetic investigation in patients will, in turn, lead to more effective treatments for bleeding and cardiovascular disease in the future.

The challenge for the clinician is to know when to send samples for genetic analysis, given the issues of interpretation. This field is still evolving but over time we will know more about our genomes and those of family members and this will play an increasingly important role in treatment and therapeutic development. Below, we have listed a sequence to aid diagnosis of a platelet function disorder, acknowledging that practices will vary between centres and countries:

- Personal bleeding history including challenges to haemostasis – surgical, dental, menstrual and childbirth
- Family history of bleeding disorders
- Medication review including drugs known to inhibit platelet function e.g. anti-inflammatories, SSRIs
- BAT score
- Exclusion of other common haemostatic disorders e.g. thrombocytopenia, von Willebrand disease, dysfibrinogenaemia with basic coagulation testing (PT, APTT and fibrinogen)
- Platelet aggregometry
- Genetic testing
- More specialist functional and genetic tests in a research laboratory

Conclusions and future developments

Given that platelets were considered to be just pieces of circulating dust about 150 years ago, they are now one of the most important cells in pathology, including both in thrombosis and thrombo-inflammatory disease. We now know the composition of the megakaryocytic/platelet genome and proteome but the challenge remains to identify which of these components are functionally important in the many roles of platelets. Progress in this area is held back by the anucleate nature of the platelet which prevents standard approaches in molecular biology. The promise of the function of large numbers of platelet genes being identified through the study of patients with platelet-based bleeding disorders has had only limited success as the majority of disorders are polygenic.

The ultimate goal of platelet research remains the development of improved antithrombotic agents that provide effective treatment without an increased risk of bleeding. Although it may never be possible to fully attain this goal, this is beginning to appear possible in certain disorders as we begin to understand more about the events that underlie thrombus formation such as the involvement of the podoplanin-CLEC-2 axis in thrombo-inflammation and FcγRIIA in VITT. The demonstration that inhibitors of tyrosine kinases used in patients with ITP and

cancer are well tolerated and causes minimal bleeding opens up the potential for repurposing as novel antiplatelets that are likely to cause less bleeding than current drugs.

Acknowledgements

SPW is a British Heart Foundation Chair. PH is the Scar Free Foundation Associate Professor. NVM is a HEFCE-funded Professor of Cardiovascular Genetics. GL is a Consultant Haematologist and Honorary Senior Clinical Lecturer. The authors gratefully acknowledge the support of the British Heart Foundation, Wellcome Trust and NIHR.

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CHAPTER 41

Haemophilia and von Willebrand disease

41

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Introduction

The existence of lifelong bleeding disorders and their familial occurrence was noted in the medical literature as early as the 16th century. Early writers were impressed by the helplessness of the physician in the face of haemophilic bleeding. Although long recognised, the pathophysiology and genetics of these disorders were not fully understood until the latter half of the 20th century. Advances in protein chemistry and molecular biology now allow a comprehensive understanding of normal coagulation, the physiological defect in haemophilia and the underlying molecular genetics. These in turn have driven new approaches to therapy. An earlier chapter outlines the normal coagulation mechanism. In this chapter, the clinical features, pathogenesis and principles of management of the most common inherited bleeding disorders, haemophilia and von Willebrand disease (VWD), are described.

Haemophilia

Pathophysiology of haemophilia

Haemophilia A and B are caused by deficiency of factor FVIII and FIX respectively. FVIII and FIX are the two components of the intrinsic tenase (see Chapter 39). Consequently, their absence causes virtually identical patterns of bleeding as well as routine blood coagulation screen results with the PT and APTT. Combined with the fact that they are both encoded on the long arm of the X chromosome, they present almost indistinguishable sex-linked clinical syndromes and specific assays are therefore required to determine which is present. In both cases, failure to form the intrinsic tenase complex results in failure to produce the thrombin burst characteristic of normal coagulation. As a result, a loose friable fibrin mesh is produced that is easily dislodged and which has increased susceptibility to fibrinolysis. Fibrinolytic breakdown of the clot is also favoured by the failure of the weak thrombin burst to activate the thrombin-activated fibrinolysis inhibitor (TAFI). The consequent failure to consolidate the primary haemostatic (platelet) plug results in the characteristic bleeding pattern of haemophilia, which is both delayed after trauma and much prolonged. Replacement by intravenous infusion of the deficient factor can normalise the haemostatic mechanism.

Clinical features

Haemophilia A and B affect approximately 1 in 10,000 and 1 in 50,000 live births respectively and are equally common in all ethnic groups. The vast majority of patients are male, but haemophilia can occur very rarely in females (see below). The severity and frequency of bleeding are inversely related to the residual level of FVIII or FIX. Table 41.1 summarises this relationship and gives the relative frequency of the categories, based on UK national data (www.ukhcdo.org). The ankles, knees and elbows are most affected but any joint can be the site of bleeding. If untreated, this intracapsular bleeding causes severe swelling, pain, stiffness and inflammation, which gradually resolves over days or weeks. It is

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 41.1 Haemophilia A*: clinical severity.

FVIII (units/dL)	Bleeding tendency	Relative incidence (%)
<1	Severe: frequent spontaneous† bleeding into joints, muscles and internal organs	50
1–5	Moderate: some 'spontaneous' bleeds, bleeding after minor trauma	30
>5–45	Mild: bleeding only after significant trauma, surgery	20

*This table is also applicable to FIX, FX and FII deficiencies, but not to FVII, FXI, FV, FXIII or von Willebrand factor deficiencies (see text).
†'Spontaneous' bleeding refers to those episodes in which no obvious precipitating event preceded the bleed. No doubt, minor tissue damage consequent on everyday activities actually initiates bleeding.

not clear why bleeding in haemophilia shows a predilection for joints and also muscles, but it has been suggested that low levels of tissue factor (TF) expression in synovial tissue may be at least part of the explanation because this makes thrombin generation more dependent on feedback amplification by the intrinsic pathway. There is some suggestion that bleeding in severe FIX deficiency is slightly less frequent. This is disputed but it may be related to the fact that genuine null mutations are more common in haemophilia A. The two disorders were not clearly distinguished until the middle of the 20th century. Haemophilia B then acquired the alternative name 'Christmas disease' after one of the boys in the first report in 1952. Bleeding into muscles and joints is the hallmark of haemophilia although the pattern of bleeding described below is now often masked by the use of prophylaxis.

Blood is highly irritating to the synovium and causes an immediate inflammatory reaction in the joint. In the longer term, blood and particularly increased iron deposition promote a chronic proliferative synovitis resulting in overgrowth of friable and highly vascular synovium which has an increased tendency to further bleeding, thus setting up a vicious cycle. As a result of the vicious cycle of bleeding and synovial hypertrophy, a particular joint frequently becomes the 'target joint' in an individual, whereas other joints may be relatively spared. Iron stimulates release of inflammatory cytokines which causes release of enzymes such as matrix metalloproteases and plasmin which degrade collagen. In addition, the cytokines promote H_2O_2 release which in combination with free Fe^{2+} generates OH^- radicals which cause chondrocyte apoptosis and loss of cartilage. The bone beneath the cartilage is also affected with osteoclast activation, bone resorption and subchondral cysts visible on X-rays. (Figure 41.1) An important observation has been that the initiation of arthropathy may require only one or two haemarthroses which means that a therapeutic strategy to prevent bleeds must be started as early as possible.

Figure 41.1 Radiograph of knee joint showing advanced haemophilic arthropathy. Note the loss of cartilage, eburnation, deformity subluxation, osteophytes, subchondral cysts and irregularity of joint contours.

Muscle bleeding can be seen in any anatomical site, but it most often presents in the large load-bearing groups of the thigh, calf, posterior abdominal wall and buttocks. Local pressure effects often cause entrapment neuropathy, particularly of the femoral nerve with iliopsoas bleeding. This causes a common symptom triad of groyne pain, hip flexure and cutaneous sensory loss over the femoral nerve distribution. Bleeding into the calf, forearm or peroneal muscles can lead to compartment syndrome with ischaemic necrosis and contracture.

Haematuria is less common than joint or muscle bleeding in individuals with haemophilia, but severely affected patients may have one or two episodes per decade. These may be painless and resolve spontaneously, but if bleeding is heavy, it can produce clot colic. Usually, no anatomical abnormality is found to account for the haematuria on radiological investigation.

Central nervous system bleeding is uncommon but can occur after minimal head injury, including birth trauma and remains

a significant cause of death in haemophilia A. Intestinal tract bleeding frequently presents with haematemesis and melaena and should be routinely investigated for peptic ulceration or malignancy. It may also present as obstruction due to intramural haemorrhage.

Oropharyngeal bleeding, although uncommon, is clinically dangerous, as extension through the soft tissues of the floor of the mouth can lead to respiratory obstruction. Bleeding from the lip or tongue after laceration can be very persistent and troublesome due to fibrinolytic substances in saliva and the impossibility of immobilising the tongue.

Surgery and open trauma invariably lead to dangerous haemorrhage in the untreated individual with haemophilia. There may be persistence of haemorrhage, often after an initial short-lived period of haemostasis. Clots, if formed, are bulky and friable and break down, with recurrent haemorrhage occurring intermittently over days and weeks. Nowadays this is only seen in patients who are resistant to conventional replacement therapy due to the presence of inhibitors (see below) or when patients with mild or moderate haemophilia present after their first surgical or dental procedure. Bruising is a common feature of haemophilia A but is usually only of cosmetic significance as it remains superficial and self-limiting. Large extending ecchymoses may occasionally require treatment.

Presentation

If a mother is a known carrier of severe haemophilia, the absence of FVIII or FIX in a cord blood sample will establish the diagnosis in the infant. It is not infrequent for prenatal genetic diagnosis to be carried out by means of amniocentesis after 36 weeks in order to guide management of delivery. Mild degrees of deficiency may be more difficult to confirm until later due to liver immaturity (FIX) or stress response (FVIII). However, haemophilia may be sporadic, and in approximately one-third of cases, there is no family history. In such cases, haemophilia may come to light in the neonatal period with cephalohaematoma or other bleeding resulting from the trauma of birth. In cultures where early circumcision is the rule, this will cause prolonged haemorrhage. Quite often, the diagnosis is delayed until it is noticed that the infant has many large bruises from hand pressure when being picked up or from minor knocks on the cot. These sometimes cause diagnostic confusion and the erroneous label of 'non-accidental injury' may be applied, with psychological trauma to the parents and child. Soon after the infant starts to walk actively, joint bleeding begins to appear. In other children, excessive bleeding from the eruption of primary dentition or from lacerations prompts performance of diagnostic tests. When there is no family history, the median age of diagnosis for severe haemophilia is 8 months. Levels of 1.0–5.0 iu/dL (moderate haemophilia) may be sufficient to largely prevent spontaneous bleeding and delay diagnosis until bleeding occurs after trauma or surgery. Mild cases may only present in adulthood when severe trauma or surgery provokes unusual bleeding.

Investigation of coagulation defects and haemophilia

Investigation of a suspected inherited bleeding disorder usually begins with routine screening tests of coagulation, which although considerably removed from normal *in vivo* coagulation mechanisms, remain useful in detecting and diagnosing coagulation disorders. The principal routine tests in common use are the prothrombin time (PT) and the activated partial thromboplastin time (APTT); also known as the partial thromboplastin time with kaolin (PTTK) or the kaolin cephalin clotting time (KCCT). These tests require the activity of all the conventional procoagulant factors except FXIII; however they do not require the presence of platelets (they are performed using platelet-poor plasma and exogenous phospholipid) and do not activate the protein C/protein S system.

Prothrombin time

In the PT, coagulation is triggered using a thromboplastin, i.e. tissue factor (TF) plus phospholipid, typically relipidated recombinant human TF or occasionally derived from animal tissue (rabbit brain). The plasma is recalcified and the clotting time recorded. The PT will be prolonged by deficiencies of FVII, FX, FV, FII or fibrinogen.

Activated partial thromboplastin time

The APPT is triggered using a negatively charged surface (e.g. kaolin, micronised silica or ellagic acid) to initiate contact activation followed by phospholipid (to mimic platelet membrane) and calcium to allow the coagulation cascade to progress. It is thought that activation of coagulation rarely proceeds by this route *in vivo* and consequently deficiency of the contact factors is not associated with any increased tendency to bleeding. The APTT will be prolonged by deficiencies of FXII, FXI, FIX, FVIII, FX, FV, FII, fibrinogen, prekallikrein and high-molecular-weight kininogen. It is, therefore, prolonged in haemophilia A and B.

Thrombin time

The thrombin time is performed simply by adding a dilute preparation of thrombin to citrated platelet-poor plasma and recording the clotting time. Prolongation of the thrombin time therefore implies that there is either an inhibitor of thrombin present (most commonly the effect of heparin) or that there is a problem with fibrin cleavage or polymerisation. It is extremely sensitive to the presence of unfractionated heparin but is also prolonged by low or abnormal fibrinogen and hypoalbuminaemia. It is normal in haemophilia.

Inhibitors

The simplest interpretation of a prolonged test of coagulation is that one or more of the factors required for its execution is deficient in the patient's plasma. However, an alternative explanation is that an inhibitor of coagulation, usually an antibody, is present. The presence of an inhibitor is typically demonstrated by mixing normal and test plasma (with a prolonged clotting time) in equal proportions. Correction of the prolonged clotting time by the normal plasma indicates a coagulation factor deficiency and failure to correct indicates the presence of an inhibitor. The mixture may need to be incubated for 2 hours for inhibitors of FVIII to manifest their effect.

When an inhibitor is detected, further tests are performed to determine whether it has specificity for a particular clotting factor or not. In practice, it may be easier to proceed directly to factor assays now they are more readily available. Non-specific inhibitors usually fall into the class of antiphospholipid antibodies and are paradoxically associated with thrombosis rather than bleeding (see Chapter 48). In previously normal patients, specific inhibitors are most commonly directed against FVIII, resulting in an 'acquired' haemophilia. In patients with haemophilia, they prevent the correction of the factor deficiency by normal plasma or therapeutically by factor concentrate.

Specific factor assays

When initial tests or clinical history suggests factor deficiency, then specific factor assays are performed to determine which is affected. This is most usually done by performing a bioassay in which the ability of the test plasma to correct the defect in plasma lacking a specific factor is measured. Bioassays have the advantage of directly measuring the biological activity of the factor in question but because they also utilise other plasma components, they are susceptible to numerous interferences and confounding effects. This can be avoided in some cases by using artificial small substrates (chromogenic substrates). This aids automation and avoids interference but often does not capture all aspects of the molecule's biological activity: for example, mutations in the *FIX* GLA domain may be missed. Finally, the physical amount of the factor present can be assessed immunologically. Immunological assays are accurate and precise but do not give information about the functional activity of the molecules that have been measured.

FVIII assays

In many laboratories, the FVIII assay is a modified APTT which is often referred to as a 'one-stage assay' because the activation of FVIII and progression to the coagulation endpoint take place in a single step. However, it is also possible to construct a 'two-stage assay' in which the FVIII is activated in a first step and its activity (the amount of Xa generated) is measured in a second separate step. Chromogenic FVIII assays also use a two-stage procedure. The two assays normally give similar results but the distinction is important because some defective FVIII molecules give different results in the two assays. Most commonly in these cases, the one-stage assay gives a higher result than the two-stage but the patient's bleeding phenotype is more in keeping with the latter (lower) result. Thus the assessment of the severity (and sometimes even the diagnosis) of the haemophilia can be mistaken if the two assays are not carried out and compared. This is referred to as 'one stage – two stage discrepancy'. The reverse phenomenon is also encountered but is less common and appears to be infrequently associated with bleeding. Factor concentrates and the products of gene therapy may also give different results with the two assays which is important when monitoring therapy.

Laboratory diagnosis of haemophilia

Initial tests show a prolonged APTT, normal PT and thrombin time (if performed) and a normal platelet count. This should prompt specific factor assays of FVIII, FIX and FXI. When a low FVIII result is encountered it should be ensured that this is not the result of von Willebrand disease.

Treatment

Clotting factor concentrates

The discovery in the early 1960s that FVIII was concentrated in cryoprecipitate and the subsequent development of lyophilised concentrates, prepared from many thousands of donors, made home treatment feasible and promised the prospect of a normal life for people with severe haemophilia. Similar concentrates containing the Vitamin K-dependent factors were developed for Haemophilia B. However, use of such plasma-derived concentrates was associated with the transmission of hepatitis C virus (HCV), hepatitis B virus (HBV) and HIV. Although these infections were present at only low frequency in the donor population, concentrates were made from many thousands of donations and there was a very high risk that batches of concentrate were infected, especially with HCV. After 1985 virucidal methods (such as terminal dry heat treatment, solvent/detergent treatment, nanofiltration and pasteurisation) were rigorously applied in conjunction with donor selection and plasma pool screening and quarantining, reducing the risk of transmitting these viruses to close to zero. However, there remains the problem of transmission of non-lipid-coated viruses (such as hepatitis A and parvovirus), which can survive solvent/detergent sterilisation and the possibility that new viruses will emerge. For these reasons, products are required to undergo more than one inactivation

process. For FIX concentrate, the process of nanofiltration has been used, which can prevent transmission of non-lipid-coated viruses. The prion protein responsible for variant Creutzfeldt–Jakob disease (vCJD) can be transmitted by blood products, but fortunately, this has not emerged as a problem in concentrates, although concentrates are no longer produced from plasma collected in countries with notable vCJD in the donor population. Consequently, the plasma-derived clotting factor concentrates in use at present are high-purity products with high specific activity and excellent safety.

Recombinant FVIII and FIX

The *FVIII* and *FIX* genes were cloned in 1984 and 1982, respectively. This has allowed the manufacture of the recombinant proteins in cell culture, which therefore eliminates the risk of disease transmission from donors. Progressive refinements have in many cases eliminated all additional animal protein from the cell culture medium and any protein added as stabiliser in the lyophilised product. Recombinant FVIII can be made either from the wild-type 'full-length' cDNA or from cDNA that has the large B domain deleted. The B domain has been shown to be completely dispensable for the procoagulant function of FVIII and its absence does not change the half-life of the molecule. Recombinant FIX is made for the wild-type 'full-length' cDNA.

Subsequently, recombinant technology has allowed the development of modified bioengineered FVIII and FIX molecules with enhanced pharmacokinetics by coupling to other molecules such as polyethylene glycol (PEG), albumin or the immunoglobulin Fc fragment. Clinical studies have demonstrated both safety and efficacy of these extended half-life (EHL) products with prolongation of plasma half-life by a factor of 1.5 for FVIII and up to 5 for FIX. This facilitates effective prophylaxis regimens and reduces burden on patients and families.

Alternatives to factor VIII and factor IX

Rebalancing therapy

Although difficult to prove, some clinical observations suggested that the bleeding phenotype was less severe in those haemophilia patients who had also inherited a prothrombotic trait such as antithrombin deficiency or Factor V Leiden. This was the stimulus for development of several therapies to deliberately lower the level of natural anticoagulant protein activity as a means of restoring the balance of coagulation rather than by restoring procoagulant protein levels. There is clear evidence that this approach is at least partially successful for all the natural anticoagulants, but the ones that have made most progress are antithrombin, TFPI and protein C. Different methods have been used for each: siRNA for antithrombin, monoclonal antibodies for TFPI and creation of a novel serpin for activated protein C. While proof of principle has been established and rates of haemarthrosis clearly reduced using these methods, their safety remains uncertain and thrombotic events have complicated the AT and TFPI studies. They all have the advantage of working for both haemophilia A and B, being unaffected by anti-FVII or anti-FIX antibodies and allowing use of novel molecules with longer half-lives which can be administered subcutaneously. Their place in therapy will rest on further safety and efficacy data.

Bispecific antibody to mimic factor VIII

The function of FVIII is to bring together FIXa and FX on a phospholipid surface and thereby increase the efficiency of FX activation by five orders of magnitude. After much effort, a bispecific human antibody was generated which was able to perform the same function. Although the antibody has a much lower efficiency than FVIII, it is able to support thrombin generation equivalent to approximately 10% of FVIII according to most estimates. Again it is not inhibited by anti-FVIII inhibitors and can be given subcutaneously at 1–4 weekly intervals making it extremely convenient for prophylaxis, without the peaks and troughs seen with concentrates and consequently extremely popular. Additional FVIII can be given to non-inhibitor patients for breakthrough bleeds or for surgery.

Treatment of bleeding in haemophilia

Since the development of replacement therapy, the goal of treatment for haemophilia patients has been the prevention of bleeding and arthropathy, and ultimately to support people with haemophilia to be able to live a life that is not impeded by haemophilia. The severity and frequency of haemarthrosis depend on both the clotting factor level and the level of activity. Only a small amount of factor activity is needed to prevent spontaneous haemarthrosis.

After trauma or at the onset of bleeding, therapeutic infusion of replacement factor should be administered as early as possible and to a haemostatic level to stop or prevent haemorrhage. The dosing of factor replacement is still based on theoretical calculations and clinical experience. Guidelines for the treatment of haemorrhagic episodes in haemophilia are given in Table 41.2, with the estimated likely effective level required for haemostasis. If sufficient factor is available, it is feasible to restore the factor level into the normal range and maintain it there by continuous infusion or frequently repeated infusions.

Formulae based on plasma volume and expected recovery give a rough guide to dosage required to achieve a given level, but where the level is critical, as for surgery or when there is serious bleeding, it should always be checked by assay after administration. On average, FVIII infusion produces a plasma increment of 2 iu/dL per unit infused per kilogramme

Table 41.2 Indications and guidelines for factor replacement in haemophilia A and B.

Site of haemorrhage	Optimal factor level (%)	Dose (units/kg body weight)		Duration (days)
		FVIII	FIX	
Joint	30–50	20–30	30–50	1–2
Muscle	30–50	20–30	30–40	1–2
Gastrointestinal tract	40–60	30–40	40–60	7–10
Oral mucosa	30–50	20–30	30–40	Until healing
Epistaxis	30–50	20–30	40–60	Until healing
Haematuria	30–100	25–50	70–100	Until healing
Central nervous system	60–100	50	80–100	7–10
Retroperitoneal	50–100	30–50	60–100	7–10
Trauma or surgery	50–100	30–50	60–100	Until healing

(*Source:* Escobar (2003). Reproduced with permission from Wiley.)

body weight, and FIX infusion a rise of 1iu/dL. From these observations, a simple formula can be derived:

$$\text{Dose to be infused}\,(\text{u}) = \text{Weight}\,(\text{kg}) \times \text{Desired rise}\,(\text{iu/dL})\,/\,\text{K}$$

where K is 1.5–2 for FVIII concentrate and 0.8–1.0 for FIX concentrate. The lower recovery for FIX reflects the fact that a substantial proportion leaves the blood and binds to collagen in the vessel wall and surrounding tissue. Some evidence suggests that this extravascular FIX makes an important contribution to haemostasis.

Assessing the duration of treatment required is a matter of clinical judgement according to the individual circumstance. Cover for surgery, other than very minor procedures, requires maintenance of normal FVIII levels for at least 1 week, followed by a period at reduced dosage during convalescence. This can be achieved either by repeated bolus injections (every 8–12 hours for FVIII and 12–24 for FIX), paying particular attention to trough levels, which should not fall below 50 iu/dL, or by continuous infusion. The half-life of FVIII varies considerably and may be particularly short in children, requiring more frequent dosing. It should also be noted that the doses required during the immediate peri- and post-operative period may be considerably more than expected. The use of EHL products can now make perioperative management considerably simpler and extend the dosing intervals quoted above.

Prophylaxis

The original rationale for prophylaxis, begun in 1958 in Malmö, was the observation that spontaneous bleeding was seen less frequently and less severely in individuals with a factor level of 1.0–4.0 iu/dL and these levels could be achieved in severe patients using regular infusions of plasma derivatives. Although this has been successful in greatly reducing the number of bleeding episodes, it has been realised that it does not completely prevent the development of joint damage which is also seen in patients with moderate haemophilia.

Today prophylaxis is regarded as the standard of care for all those with severe haemophilia and should also be considered for the majority of those with moderate haemophilia. Regular prophylactic treatment is begun at around the age of 1 year, before the onset of joint bleeds, so-called primary prophylaxis. Standard half-life FVIII is administered every second day at a dose of 25–40 units/kg. The goal is to adjust the dose and frequency to maintain peak and trough levels that fit with the patient's activity of lifestyle and prevent all bleeding episodes. Evidence from trials indicates that the risk of bleeding rises as the length of time spent with FVIII levels below 1.0 iu/dL increases. Historically, the Malmö model was effective in reducing haemophilic arthropathy. (Figure 41.2). More recently, a large, randomised study comparing prophylaxis with enhanced on-demand therapy showed that after 6 years of therapy, 93% of those on primary prophylaxis had a normal joint score on magnetic resonance imaging (MRI) compared with only 55% on intensive on-demand regimens. However, as protection from bleeding improves, so levels of activity increase and the intensity of treatment has to be increased in parallel to match this. Regrettably, children on apparently effective prophylactic regimens are still entering their third decade with significant arthropathy, frequently in the ankles and having to undergo joint fusion. In some cases, joint damage has developed without overt bleeds leading to the hypothesis that subclinical 'microbleeds' may be occurring, although this remains unproven. The current goal of prophylaxis is to achieve an annualised bleeding rate of zero but achieving this as well as a full range of activity still incurs a considerable treatment burden and requires multidisciplinary support.

Factor concentrates must be delivered via a peripheral vein for prophylaxis. However, if this is not possible then the implantation of an indwelling venous access device (e.g. Port-a-Cath)

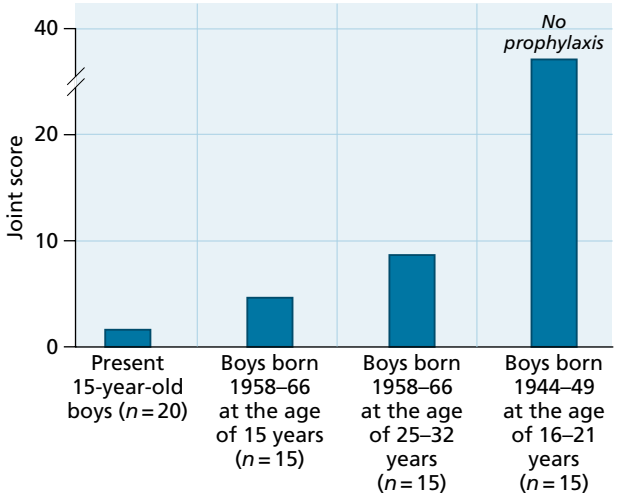

Figure 41.2 Orthopaedic joint scores for patients intensively treated in the present. A group of 15-year-old boys compared with a less intensively treated group at different ages and with patients not receiving prophylaxis (historical control subjects) (Source: United Kingdom Haemophilia Centre Doctors' Organisation (2003). Reproduced with permission of Wiley.)

may be necessary. Another alternative is the fashioning of a small arteriovenous fistula in the arm. EHL concentrates can reduce the need for this by allowing longer intervals between treatment. At present, this is every third day for haemophilia A and weekly or fortnightly for haemophilia B, but new products may extend this further. Use of EHLs has also increased levels of activity and quality of life. The bispecific antibody can simplify prophylaxis further for haemophilia A by providing good protection from bleeds (70% of patients with zero bleeds), injections 1–4 weekly and subcutaneous administration. The benefit is more dramatic in patients with inhibitors to FVIII (see Section below) for whom there was previously no adequate prophylactic therapy.

In older patients who have established joint disease or have had previous joint bleeds, prophylaxis is also effective in reducing bleeding episodes and/or slowing progression and even improving joint disease. This 'secondary or tertiary' prophylaxis may be used continuously or for a limited period to allow the synovial hypertrophy in a target joint to regress or be targeted to periods when bleeding is more likely (such as a particular activity).

Tranexamic acid

A significant component of the bleeding tendency in haemophilia is the increased susceptibility of the clot to fibrinolysis. Tranexamic acid is a lysine analogue that competes with fibrin for binding to the kringle domains of plasminogen thus preventing its binding to fibrin and inhibiting its conversion to plasmin. This anti-fibrinolytic effect makes tranexamic acid a useful adjunctive therapy in haemophilia, especially for bleeding at sites of increased fibrinolytic activity such as the oral mucosa. It can be given orally or intravenously, ideally beginning before surgery, at a dose of 1 g tds (20 mg/kg/day for children in divided doses). For oral bleeding, a mouthwash can be prepared (5% w/v) which is also useful. Dose adjustment is required in renal impairment.

Desmopressin

Desmopressin (1-deamino-8-d-arginine vasopressin) can be used to treat mild haemophilia A and provides a non-plasma alternative to concentrates. It has no effect on FIX deficiency. It acts via the V_2 receptors on endothelial cells to stimulate exocytosis of Weibel–Palade bodies which contain VWF and, in some tissues, also FVIII. In practice, this effect can be used to elevate the plasma FVIII level two to fourfold above baseline. Desmopressin is a synthetic analogue of vasopressin and retains the antidiuretic action of the natural hormone but not it's vasopressor activity. Patients are advised to restrict their fluid intake after desmopressin. This is especially important in young children in whom unrestricted fluid intake may result in hyponatraemia and convulsions. Desmopressin is contraindicated in elderly patients and those with vascular disease because arterial thrombosis has been reported following desmopressin in these circumstances.

Desmopressin can correct the haemostatic defect in mild haemophilia A sufficiently to cover minor surgery or treat a minor bleeding episode. A typical regimen would be to give 0.3 μg/kg body weight either by slow intravenous infusion over 20 min or by subcutaneous injection. Desmopressin also releases tissue plasminogen activator and so tranexamic acid is frequently given as well. A high-concentration nasal spray is also available that delivers 150 μg per actuation. The effect is maximal at approximately 60 min after either intravenous or subcutaneous administration. The half-life of the endogenously released FVIII is about 8 hours but may be shorter in some cases and the fall-off should be documented. The dose can be repeated and although tachyphylaxis occurs such that the second dose may produce a rise of only 30% of the first, there is often no further drop with succeeding doses. If repeated doses are given over a short interval (e.g. 48–72 hours), the FVIII response to desmopressin injection should be assayed to ensure that the reserves are not exhausted.

Complications of therapy

Inhibitors

Antibodies to the FVIII replacement (inhibitors) are most common in patients with severe Haemophilia A. In people with severe Haemophilia A they are most likely to develop during

childhood within the first 25 exposures to FVIII; after which they are infrequent, but do rarely develop after years of exposure, typically after periods of intensive FVIII treatment. Inhibitors are detected in around 25–30% of people with severe haemophilia A at some stage in their treatment. However, many of these are low-level transient inhibitors that are not a long-term problem for treatment. The cumulative incidence of high-responding inhibitors is in the region of 10–15%. The likelihood of an individual developing an inhibitor is strongly dependent on the underlying *FVIII* mutation. Patients with large deletions or stop codons in the light chain appear to be particularly prone to inhibitor development. Approximately 25% of patients with the common intron-22 inversion develop inhibitors.

Inhibitors in moderate and mild haemophilia are far less common. However, some missense mutations causing milder haemophilia A, particularly those in the FVIII light chain, are strongly associated with inhibitor development. Typically such patients develop inhibitors when FVIII is administered in large amounts (e.g. severe bleeds) or in association with inflammatory stimuli (e.g. surgery). Because the development of inhibitors is unpredictable, it is important to test for their emergence at clinic visits, and before and after operations in all patients that have been exposed to FVIII concentrates.

Antibodies to FVIII frequently have a time-dependent component in their action and an incubation time of 2–4 hours is required to reliably measure the full effect of the antibody. Inhibitors usually inactivate FVIII in a predictable linear manner (type 1 kinetics) but they may not inactivate all available FVIII (type 2 kinetics). Type 2 kinetics are seen particularly in mild and moderate haemophilia as well as in acquired haemophilia. Inhibitors are most commonly quantitated using the Bethesda assay where 1 Bethesda unit (BU) is the amount of antibody that reduces the FVIII activity in pooled normal plasma by 50% after 2 hours incubation. Some inhibitors are 'low-responding' and remain at a low or moderate level (<5–10 BU) after further FVIII exposure, whereas in high responders treatment with FVIII, elicits a sharp anamnestic rise after 5–8 days and inhibitor levels may reach hundreds or thousands of BU. Low responders can be treated repeatedly with high doses of FVIII concentrate. However, high responders are refractory to treatment with FVIII concentrate, and therefore alternative therapies are required.

Inhibitors in haemophilia B are much less common than in haemophilia A. Only about 2–3% of patients with severe haemophilia B will develop inhibitors, but interestingly many of these inhibitors present with anaphylaxis to infusions of FIX concentrate, sometimes after only one or two exposures. Unlike FVIII inhibitors, FIX inhibitors are not time-dependent, but can be similarly quantitated by a modified Bethesda assay. The majority of patients who develop inhibitors have large gene deletions or nonsense mutations occurring in the first 20% of the *FIX* gene.

Inhibitors: treatment and prevention of bleeding

For patients with Haemophilia A and antibodies to FVIII, effective prophylaxis is now available using the bispecific antibody which is as effective as for non-inhibitor patients. There is no such FIX substitute for patients with HB although the rebalancing therapies offer the prospect of a suitable prophylactic agent.

For treatment of bleeding episodes or for surgery, a bypassing agent will often be required to achieve haemostasis. The currently available bypassing agents are human recombinant FVII (rFVIIa) and a plasma-derived prothrombin complex concentrate that contains activated coagulation factors (factor eight inhibitor bypassing activity, or FEIBA). The mode of action of rFVIIa is not clear but it appears to bind to the surface of activated platelets at the site of injury and promotes FXa formation there. Recent evidence suggests that platelets may be able to synthesise TF or at least acquire it from circulating microparticles. High concentrations (30–90 nmol/L) of FVIIa are required to generate sufficient FXa and FIIa to achieve haemostasis. The advantages of recombinant FVIIa include viral safety, low systemic activation of coagulation, effectiveness independent of inhibitor titre, and an excellent overall safety profile allowing home use and ease of administration. The main disadvantage is the short 2-hour half-life and therefore the need for frequent administration at standard dose (90 mcg/kg), although this may be partially overcome by using a single 270 mcg/kg dose for mild to moderate bleeds.

Both native and activated prothrombin complex concentrates appear to be able to bypass the need for FVIII and have a role in the treatment of patients with FVIII antibodies. Their efficacy appears to derive from a combination of activated clotting factors and the elevation of prothrombin concentration, which is a major determinant of thrombin generation. The activated prothrombin complex concentrates (APCC) contain more of the FVIII bypassing activity and are designed specifically for use in patients with FVIII inhibitors. Activated prothrombin complex concentrates have proved efficacious in controlling bleeding in many situations. Currently, the principal problem with APCC is that when used in combination with the bispecific antibody, it can produce a syndrome of microangiopathy and thrombosis. It is recommended that this combination is avoided. Other problems with APCC are that it is plasma-derived and it contains some FVIII, which may cause an anamnestic rise in inhibitor titre. On the other hand, its longer dosage interval (given once or twice daily) makes it simpler to use. A major problem with both bypassing agents is that there is no reliable laboratory measure of their haemostatic effect.

Treatment of inhibitors: immune tolerance

Until alternative molecules capable of reproducing normal levels of FVIII or FIX activity are developed, it will remain desirable to eradicate FVIII or FIX inhibitors, a process referred to as immune tolerance induction (ITI).

FVIII inhibitors: For haemophilia A basic ITI regimens involve the continued administration of FVIII, and both low-dose (50 units/kg 3 times per week or alternate days) and high-dose (200 units/kg/day) regimens have been used. Neither regimen has been conclusively shown to be superior but the response is more rapid and bleeding events are fewer, in the high-dose regimen. Both may need to be continued for 12 months or more. Overall, the success rate is approximately 70%, after which prophylaxis must be maintained to prevent re-emergence of the antibody. The success is greatest in those with a historic peak titre below 200 BU, and where there are no interruptions to the ITI regime. For resistant cases, second-line approaches include the use of a FVIII concentrate containing VWF (for FVIII inhibitors), combination with monoclonal anti-CD20 antibody, or corticosteroids, alkylating agents, plasmapheresis and immunoglobulin (Malmö regimen). Success is defined as normalisation of the FVIII recovery and a FVIII half-life greater than 7 hours. Following successful immune tolerance, if regular FVIII prophylaxis is stopped, for example in order to use the bispecific antibody as subcutaneous prophylaxis, there is a risk of immune tolerance 'escape' and the FVIII inhibitor returning; this decision therefore needs to be considered very carefully.

FIX inhibitors: Immune tolerance for FIX inhibitors is generally less successful and is sometimes precluded by anaphylactic responses making more elaborate regimens necessary beginning with very low doses of FIX and administration by continuous infusion. It may also be complicated by the development of nephrotic syndrome.

The ageing haemophilia population

Although HIV and HCV remain significant complications of therapy, improvements in both haemophilia care and antiviral treatments have resulted in significant increases in median life expectancy for individuals with haemophilia over recent years. Increasing numbers of patients are living beyond 65 years of age. In addition to the joint and muscle problems of haemophilia, older patients face the same medical conditions associated with ageing in the general population, such as cardiovascular disease and cancer. Care can become very complex, such as use of antithrombotic therapy for cardiovascular disease in a patient with an inherited bleeding disorder. For example, intensive prophylaxis to maintain FVIII above 0.3–0.5 iu/mL may be required to allow anticoagulation for AF or dual antiplatelet therapy following coronary stent placement. Multidisciplinary and cross-specialty coordination is key to successful management.

Molecular genetics of haemophiia A

The *FVIII* gene (Figure 41.3) spans 190 kb of the X chromosome. The protein-coding regions (exons) are separated by 25 introns, some of very large size (e.g. intron 22 is over 35 kb). FVIII is synthesised in endothelial cells especially in the liver, spleen and lymph nodes. The amino acid sequence contains a triplicated region (A1, A2 and A3 in Figure 41.3), whose elements are more than 30% homologous with each other. A second duplicated homology region (C1, C2) is responsible for the phospholipid-binding properties of the molecule. The third type of sequence in the protein is the heavily glycosylated B domain, which is coded entirely within exon 14, connects A2 and A3, and is removed on thrombin activation of FVIII.

The processed mRNA specifies a protein of 2351 amino acids, from which a 19-amino-acid N-terminal leader sequence is cleaved on secretion. The mature plasma protein initially consists of a single chain of 2332 amino acids, but as a result of proteolysis circulates as a series of light-chain and heavy-chain heterodimers with B domains of varying length.

The vast majority (>90%) of FVIII circulates in complex with VWF, in the absence of which its half-life is extremely short. Studies of FVIII have identified the A3 (1648–1689), C1 and C2 domains as important for VWF binding but it is uncertain how many of these interact directly with VWF because in isolation the recombinant fragments have a much lower affinity. After thrombin activation, the cofactor consists of a heterotrimer: the N-terminal A1 and the A2 domains are isolated by cleavages at 372/373 and 740/741, and held together by charge interactions including a divalent cation-dependent linkage (see Figure 41.3). The B domain is discarded by the cleavages at 740/741 and 1689/1690. Thrombin cleavage also results in release of FVIII from VWF.

Protein C inactivates FVIIIa by cleavage at 336/337 and 562/563 totally but *in vivo* inactivation of FVIIIa is also due to spontaneous dissociation of the heterotrimer. The crystal structure of FVIII has been reported and several of the interactions mapped including the FIX-binding site on the A2 and A3 domains. The C domains appear to be responsible for phospholipid binding.

Thanks to the development of rapid sequencing methods, the mutations in nearly all patients with haemophilia A can be identified (Table 41.3). Consequently, a large database of mutations has accumulated and is maintained at https://databases.lovd.nl/shared/genes/F8. These include large deletions and missense mutations, as well as nonsense, frameshift, splicing (affecting mRNA processing) and insertional mutations. Surprisingly, about half of all severe haemophilia A is due to a major inversion (Figure 41.4) that occurs quite frequently during male gametogenesis (approximately 1 in 10,000 spermatozoa).

Molecular genetics of haemophilia B

The *FIX* gene lies telomeric to the *FVIII* gene on the X chromosome at Xq27.1 and is considerably smaller extending for only 34 kb in length. It comprises eight exons producing

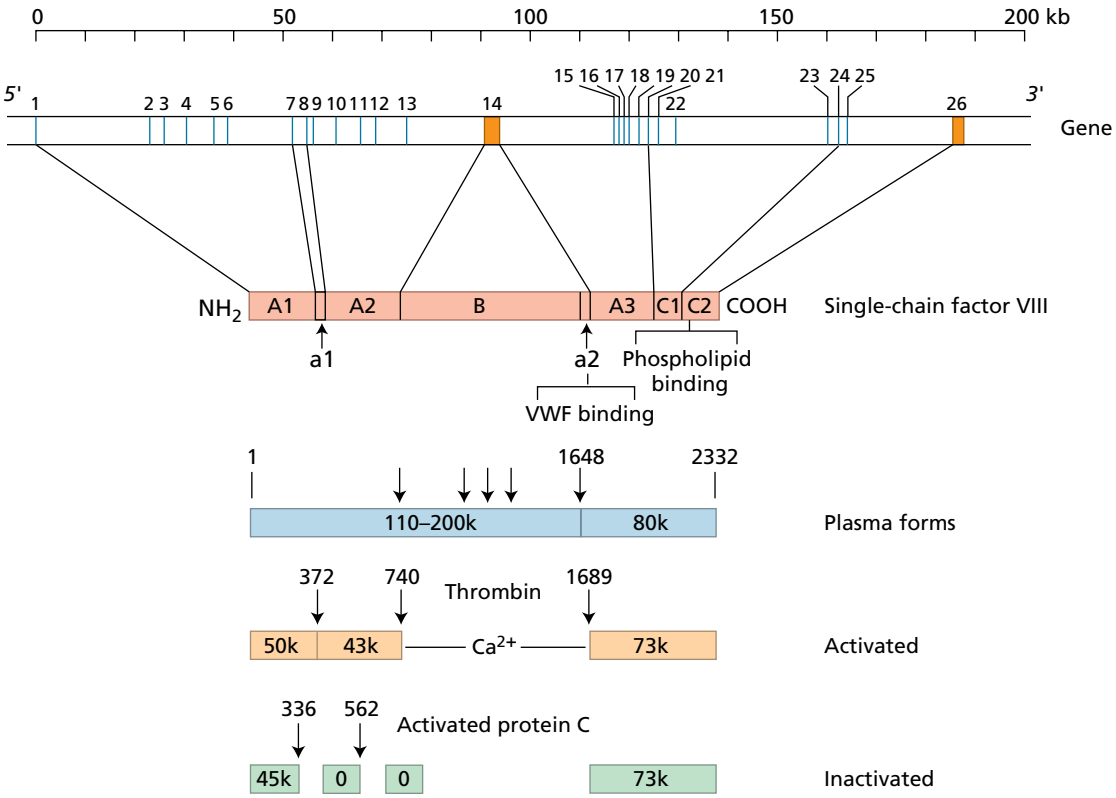

Figure 41.3 The *FVIII* gene and protein. Top line, scale for gene in kilobase pairs of DNA; second line, location of exons (protein-coding segments) of the *FVIII* gene shown as solid bars; second to third line, corresponding domains of *FVIII* gene and protein; fourth line, plasma FVIII is heterogeneous due to cleavage at various points within the B domain during synthesis and secretion, yielding heterodimers in which the heavy chain (N-terminal segment) pairs from 740 to 1648 amino acids; fifth line, thrombin activation releases the a2 segment from the light chain, the B domain from the heavy chain and cleaves between the A1 and A2 domains; bottom line, inactivation of factor VIIIa by activated protein C is accompanied by cleavages at 336, releasing the a1 domain and 562 in the middle of the A2 domain.

a 2.8 kb transcript mRNA encoding the protein of 461aa. FIX is synthesised in the liver and has a domain structure similar to the other serine proteases of the coagulation cascade, comprising a signal peptide, a GLA domain, two EGF domains and a serine protease domain. The single-chain zymogen can be activated by both FXIa and the TF-VIIa complex; in both cases, two cleavages after Arg 191 and Arg 226 release an activation peptide creating a disulfide-linked two-chain active enzyme. The mutation spectrum responsible for Haemophilia B is quite different from that responsible for Haemophilia A primarily because there is no single mutation corresponding to the intron 22 inversion. Instead, the majority of mutations responsible for severe Haemophilia B are missense mutations rather than nonsense mutations as for Haemophilia A (https://databases.lovd.nl/shared/genes/F9). This may account for any difference in phenotype but again it is large deletions that are associated with the highest risk of inhibitor formation. The normal plasma concentration of FIX (~90 nM) is much higher than that of FVIII (~0.4 nM).

Gene therapy for haemophilia

Haemophilia is an excellent model for gene therapy (GT) because the clinical manifestation is the result of a deficiency of the single gene product and only a small amount of protein is required to ameliorate symptoms. Providing the protein enters the blood, the site of synthesis should not matter. In recent years a series of trials using *FVIII* and *FIX* genes in adeno-associated virus (AAV) vectors have achieved successful and long-lasting expression with resultant reduction in bleeds and concentrate requirement, often with cessation of prophylaxis. The AAV viruses have relative tropism for the liver and the construct typically contains a liver-specific promoter for expression in that organ. This is the physiological site of synthesis for FIX but not for FVIII which may contribute to differences in the patterns of expression.

The first studies dealt with FIX due to the smaller size of the gene and its lower immunogenicity. Gene therapy for FIX has

Table 41.3 Mutations responsible for haemophilia A.

Mutation type	No. of mutations observed	Percentage
Intron 22 inversion		
Distal	260	30.7
Proximal	37	4.4
Rare	5	0.6
Total	302	35.7
Intron 1 inversion		
Total	8	1.0
Point mutations		
Missense	323	38.2
Nonsense	79	9.3
Total	402	47.5
Small deletions or insertions		
Small deletions	63	7.5
Small insertions	22	2.6
Combination of insertions and deletions	1	0.1
Total	86	10.2
Large deletions (>50 bp)		
Total	25	3.0
Splice-site mutations		
Intronic	20	2.4
Cryptic	2	0.2
Total	22	2.6

(*Source:* Graw *et al.* (2005). Reproduced with permission from Nature Publishing.)

been enhanced by utilising the FIX Padua variant which has a specific activity approximately 8 times that of wild-type FIX allowing higher levels of FIX activity to be achieved, often in the normal range, with relatively low levels of protein expression and vector doses.

Gene therapy for FVIII is more difficult because even after deletion of the B-domain the gene construct remains slightly too large to fit within AAV and so multiple transfection of cells with partial constructs is required. GT for both FVIII and FIX has been complicated by immune reactions to the viral capsid and possibly innate immune responses which manifest as liver enzyme elevation and loss of protein production. Most GT recipients have been treated with temporary immunosuppression to suppress these reactions. At present, most gene therapy programmes utilise AAV which are non-integrating episomal viruses but other approaches such as haematopoietic stem cell transduction, and vectors such as lentivirus, remain in development. The significance of rare integration events is not yet clear but has not resulted in any problems so far.

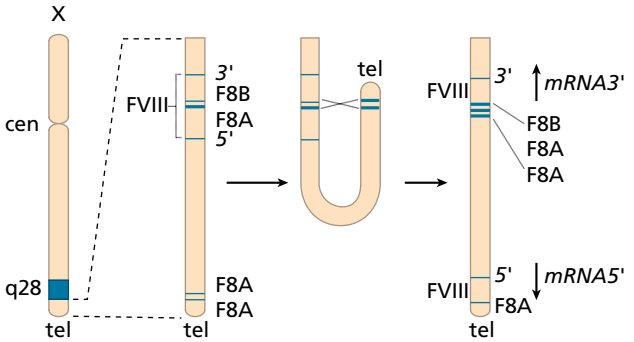

Figure 41.4 How the tip flips: the mechanism of inversion through intron 22. The mutation is responsible for up to half of all cases of severe haemophilia A. During spermatogenesis, at meiosis the single X pairs with the Y chromosome in the homologous regions, but there is nothing to pair with most of the long arm of X. Unfortunately, the possibility of intrachromosomal pairing and crossover exists because there are three copies of a gene designated *F8A*, one lying within intron 22 and two in the opposite orientation situated 400 kb telomeric to the *FVIII* gene. Crossover with either the distal or the proximal *F8A* copy divides the *FVIII* gene in two, such that separately transcribed mRNAs are produced, neither of which encodes functional FVIII.

Haemophilia A and B in females

True homozygous haemophilia A is rare and even rarer for haemophilia B, but well described, being due to the marriage of a female carrier of haemophilia to an affected male. Haemophilia A and B have also been described in females with Turner syndrome.

More commonly, carriers of haemophilia may have haemophilia themselves due to skewed X chromosome inactivation (Lyonisation). X-chromosome inactivation allows only one *FVIII/FIX* gene to function in each female cell. Because the process is random, carriers of haemophilia A or B have on average 50% of the mean normal level of the clotting factor but skewing of the inactivation towards the X chromosome containing the abnormal or the normal *FVIII/FIX* gene may occur, resulting in a clotting factor level that is either well within the normal range or low enough to produce a mild, or even moderate, haemophilia phenotype. In this case, bleeding is similar to males with the same factor level with the additional problems of HMB and pregnancy and responds to factor replacement. It is important to note that while a low level of FVIII/FIX strongly implies carriership, a normal FVIII/FIX level does not exclude it.

As a result of *de novo* mutations, especially the FVIII intron 22 inversion in spermatogenesis, new cases may present with no family history of haemophilia. In such women, careful investigation is essential to ensure the correct diagnosis is made – haemophilia carrier, mosaic, VWD type 1 or VWD type 2N (see below) – due to the reproductive implications. Molecular analysis is often required to clarify the diagnosis.

Current techniques make definitive diagnosis by identification of the causative mutation in patients and carriers straightforward in the vast majority of cases. In identified carriers, antenatal diagnosis can be carried out when pregnant and although typically only offered in cases of severe haemophilia specific diagnosis can be made by molecular analysis of chorionic villus biopsy at 11–12 weeks' gestation or third-trimester amniocentesis. The sex of the fetus can be determined from free fetal DNA in maternal blood at 8–9 weeks gestation. Pre-implantation genetic diagnosis (PIGD) with selection of unaffected embryos for reimplantation has been successfully carried out for haemophilia.

General organisation of haemophilia care

As these are relatively uncommon disorders, with effects on patients and families at all stages of life who require care and support services across the whole field of medicine and social services, it is now accepted that this is best delivered by specialised comprehensive care centres. The staff of a major comprehensive care centre will include physicians, nurses, physiotherapists, psychologists, laboratory scientists and social workers, devoting all or a substantial part of their time to haemophilia care. Key link specialties, where often joint clinics may be held include obstetrics, gynaecology, rheumatology and orthopaedics. Access to specialised care for patients with HIV and HCV infection is still important.

Within the NHS, a service specification has been developed to support and set out the provision of haemophilia services. It is recognised that not every centre can provide every facility and that there should be a wide distribution according to population density. The functions of a centre are to provide 24-hour emergency treatment for haemophilic patients and their families and a full range of diagnostic tests for identifying new patients and monitoring treatment. Full records should be kept of all treatments, whether given in hospital or as home therapy and progress monitored through regular follow-up. Paediatric centres are best located within paediatric units. Genetic counselling, including carrier detection and antenatal diagnosis, must be available for families of patients with haemophilia. All patients should have direct or indirect access to a large centre providing all these facilities and treating a large number of patients. On diagnosis, all patients are issued with a special medical card indicating laboratory test results, inhibitor status, main centre and local centre for treatment. Despite the setbacks in the 1980s due to virus transmission, the trend continues to be an ever-improving life expectancy and social participation, based on continuing medical progress and multi-professional input. The younger generation of people with haemophilia is now treated with virus-safe or recombinant concentrates and has escaped both HIV and liver disease, and can lead virtually normal lives

Acquired haemophilia

This chapter is devoted primarily to the inherited form of haemophilia arising from mutations in the *FVIII* and *FIX* genes but haemophilia can also arise as a result of autoantibodies that neutralise FVIII cofactor activity. The incidence is approximately 1.5 per million per year and it tends to occur predominantly in older patients, although there is also an increased frequency after pregnancy and in association with other autoimmune disorders. The antibodies usually show time-dependent (type 2) neutralisation of FVIII and a residual FVIII level is often detectable. The pattern of bleeding is distinct from that in inherited haemophilia, with a much greater incidence of soft tissue and mucosal bleeding, often out of proportion to the FVIII level. Treatment for bleeding is generally the same as for inhibitors in hereditary haemophilia and suppression of the inhibitor requires immunosuppressive therapy (e.g. corticosteroids, cyclophosphamide and anti-CD20 antibody). In some cases, a suitable alternative for haemostasis is porcine FVIII which functions well in human plasma but is frequently neutralised less efficiently by human anti-FVIII antibodies, especially in acquired haemophilia. A recombinant porcine FVIII has been developed and licensed for use when antibody cross-reactivity is demonstrated to be low. It has the advantage that the response can be measured using a FVIII assay and so indicate the efficacy of treatment.

von Willebrand disease

Although described in 1926, our understanding of this complex and variable bleeding disorder remains far from complete. The basic defect common to all variants is a deficiency of one or more aspects of VWF functional activity. The abnormality may be quantitative and/or qualitative.

von Willebrand factor

von Willebrand factor (VWF) is a large plasma glycoprotein encoded by a gene on chromosome 12p13, which was cloned simultaneously by several groups in 1986. The pre-pro-VWF primary translation product comprises a 22-amino-acid signal peptide, a 741-amino-acid propeptide and the 2050-amino-acid mature subunit. The pro-VWF monomer is now described as comprising three types of domains (A, C and D) arranged as follows: NH_2–D1–D2–D′–D3–A1–A2–A3–D4–C1–C2–-C3-C4-C5-C6-CK–COOH. (Figure 41.5) These can themselves be broken down into smaller ancestral units. VWF is produced predominantly in vascular endothelial cells and also in megakaryocytes, although plasma VWF derives entirely from endothelial cell production. After translation, the monomer enters the endoplasmic reticulum and the signal peptide is removed. The monomers then dimerise via the C-terminal

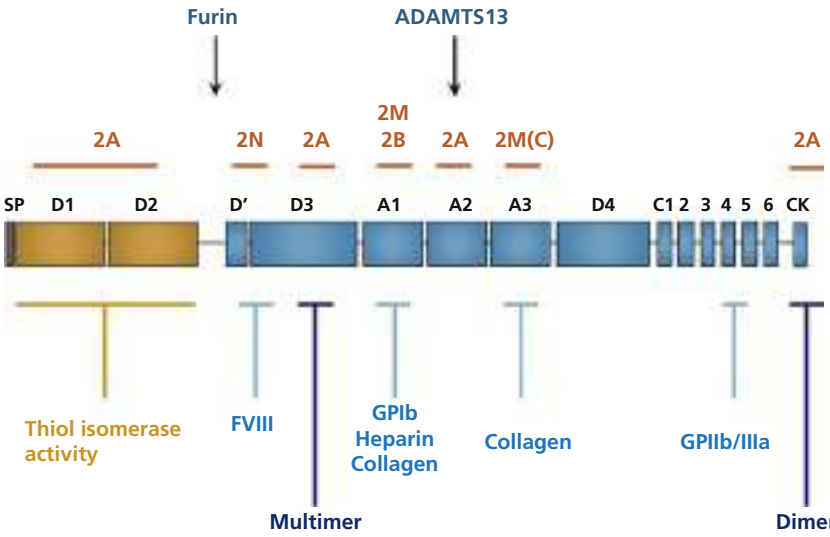

Figure 41.5 The domain structure of the primary VWF translation product. The SP-D1D2D′D3A1A2A3D4C1-C6CK domain structure is shown with the binding properties of the domains indicated below including the sites involved in dimerisation and multimerisation. Variants responsible the different type 2 variants cluster in the are shown above the domains. Variants responsible for type 1 and type 3 are distributed throughout the molecule and are not shown. Cleavage by Furin removes the propeptide before or soon after secretion. The ADAMTS13 cleavage site in the A2 domain is also shown. SP, signal peptide; CK, cysteine knot. 2M(C) indicates a type 2M pattern caused by defective collagen binding.

cysteine knot (CK) domain before leaving the endoplasmic reticulum for the Golgi, where the dimers are joined via their N-termini to form a series of multimers (or more strictly concatemers) with molecular masses ranging from 1000 to 20,000 kDa. Following translation and during subsequent processing the molecule is extensively glycosylated with N- and O-linked glycans that form 20% of its final molecular weight. Importantly, approximately 13% of the N-linked glycans terminate in an ABO blood group antigen. In endothelial cells, multimerised VWF is then tightly packed in helical structures within Weibel–Palade bodies, some of which pass directly to the cell surface to release their contents (constitutive pathway) while others are delayed in the cell for later release or in response to inflammatory stimuli (regulated pathway). Platelet VWF does not contribute significantly to plasma VWF and is stored in the α-granules of platelets prior to release upon platelet activation. During synthesis, the VWF propeptide, which is essential for multimerisation, is cleaved from the mature molecule, stored with VWF in the Weibel–Palade bodies and released with it into plasma but is not known to have any further function thereafter. Some VWF is also secreted from the base of the endothelial cell where it binds directly to collagen in the subendothelial matrix. Factor VIII (FVIII) is also synthesised in endothelial cells and stored with VWF in the Weibel-Palade bodies.

Rapid release of VWF and FVIII from the endothelial Weibel–Palade storage granules via the regulated pathway can be induced by a number of agonists including thrombin, adrenaline, histamine and vasopressin. This can be turned to advantage by using desmopressin to treat mild forms of VWD. Elevation of plasma VWF due to increased synthesis and secretion occurs as part of the acute-phase response to injury, inflammation, infection and neoplasia and also in pregnancy and hyperthyroidism. The confounding effects of stress and inflammation need to be taken into account when measuring VWF levels for diagnosis.

The two principal functions of VWF are (i) adhesive, binding to matrix molecules, particularly collagen, at sites of vascular injury and subsequent capture of platelets to form the primary haemostatic plug and (ii) the stabilisation of FVIII in the circulation. The adhesive function of VWF requires functional binding sites for collagen and the platelet glycoproteins GPIb and GPIIb/IIIa (also known as integrin $\alpha_{IIb}\beta_3$) and is also dependent on the presence of large VWF multimers. The VWF released from endothelial cells into plasma is in the form of ultra-large multimers, which are subsequently cleaved into smaller forms by the action of the plasma metalloprotease ADAMTS-13. This is of critical importance because it is the high-molecular-weight multimers that have the greatest collagen- and platelet-binding activity: absent cleavage allows ultra-large multimers to persist in the circulation resulting in the formation of platelet microthrombi and vascular occlusion (in thrombotic thrombocytopenic purpura; TTP). Conversely, excessive cleavage results in the loss of high-molecular-weight multimers, loss of haemostatic function and increased bleeding as seen in some forms of acquired von Willebrand syndrome and some type 2A VWD (see 'classification of VWD' below).

VWF also binds to a number of other molecules present in the Weibel–Palade bodies such as osteoprotegerin and angiopoietin 2 but the functional importance of this is not entirely clear, although VWF is now known to play an important role in angiogenesis and wound healing.

FVIII and VWF (Figure 41.6) circulate together as a complex in which VWF protects FVIII from degradation, so that a deficiency of VWF or a reduction in its ability to bind FVIII may result in a low FVIII plasma level. Therefore, deficiency of VWF can give rise to a dual haemostatic defect: reduced plasma levels of FVIII (due to its shorter half-life in the absence of VWF) and a defect in primary haemostasis because of the failure in assisting platelets to adhere to the cut edges of blood vessels (see Chapter 40). Clearly, the multistep synthesis, assembly and secretion of VWF and its multiple binding

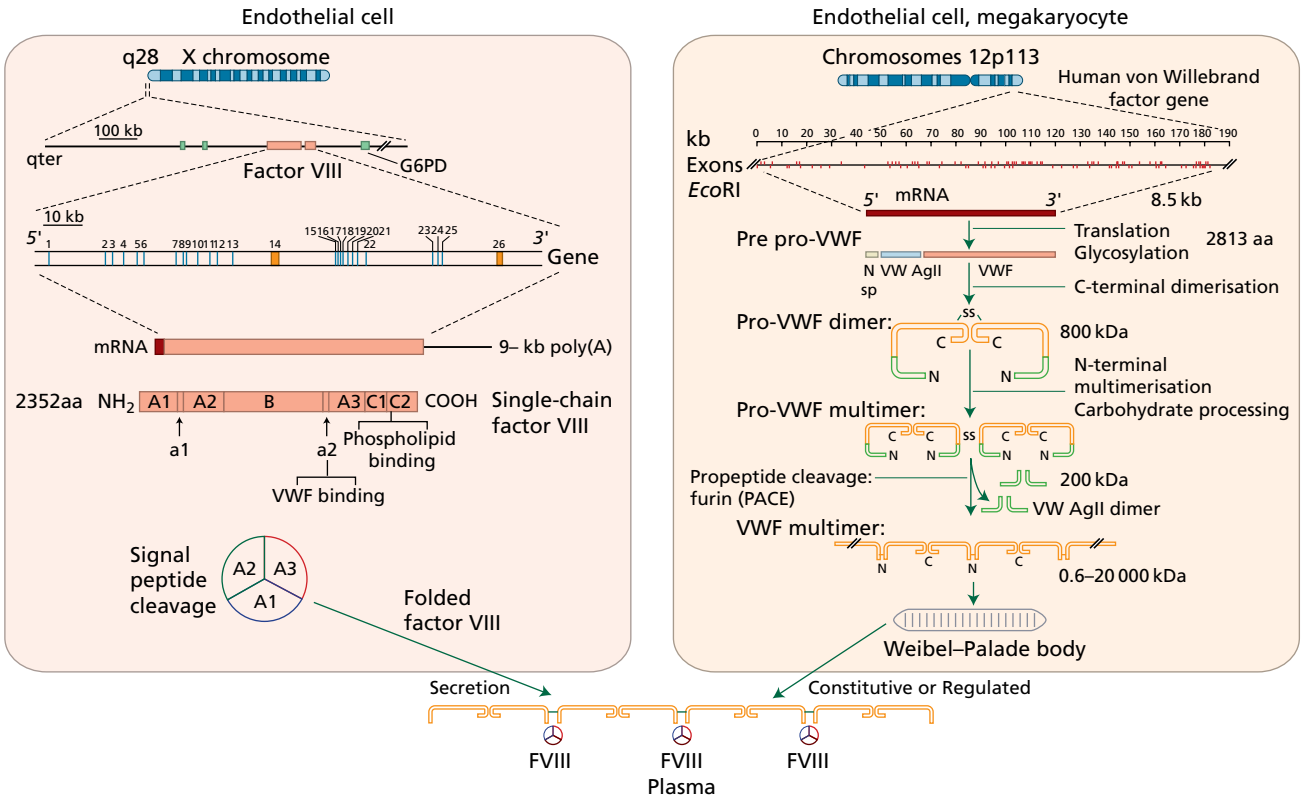

Figure 41.6 Assembly of FVIII–VWF complex. FVIII synthesised by endothelial cells as a single chain is partially proteolysed and probably complexed with VWF prior to release. VWF is synthesised as a single-chain precursor, which dimerises with disulfide bond formation, then multimerises with further disulfide exchange, with concomitant loss of a large propeptide segment. The propeptide is essential for multimer formation and probably functions as an acidic disulfide isomerase. In megakaryocytes VWF is stored in alpha granules.

interactions provide many ways in which mutations of the *VWF* gene can lead to loss of function. In particular, the multimeric structure is susceptible to dominant-negative effects of mutant alleles which can result in premature multimer termination. Genome-wide association studies have identified many genes which contribute to determining the plasma VWF level either by modifying synthesis, storage and secretion or by modifying clearance from the circulation. Chief amongst these is the *ABO* locus which is responsible for approximately 20% of the variation, with blood group O individuals having VWF levels 30% lower than those who are non-O.

The diagnosis of VWD is based on the recognition that for normal VWF function, it must be (i) present in adequate amounts, (ii) have a normal multimeric structure and (iii) have intact functional domains (binding sites). When any of these properties is only slightly reduced, the presence or absence of a bleeding tendency may also depend on the quality and quantity of the other haemostatic components, particularly platelets and collagen, with which VWF must interact to complete the process of primary haemostasis. This 'net effect' problem is the likely explanation for the variable phenotype associated with mildly low levels of VWF and the associated diagnostic confusion.

Clinical features

The clinical features of VWD vary according to the severity of the deficiency. The most common clinical picture is of a mild to moderately severe bleeding tendency characterised by bruising, epistaxis, prolonged bleeding from minor cuts, heavy menstrual bleeding and excessive, but not often life-threatening, bleeding after trauma or surgery. Patients often present for investigation in the second or third decade after prolonged bleeding from dental extraction or surgery has aroused clinical suspicion. Heavy menstrual bleeding (HMB), unexplained by local or hormonal factors, can also be the presenting symptom: an estimated 20% of women with HMB have VWD and data suggest this is widely underappreciated and under-investigated.

The distribution of bleeding in VWD can be explained on the basis that VWF is required for platelet adhesion at high shear rate, which is the condition of flow found in the smallest blood vessels exposed to trauma in skin and mucous membranes. Type 2A VWF

is particularly associated with bleeding from the gut which may be complicated by the development of angiodysplasia. Much less common is autosomal recessive (type 3) VWD, where VWF is completely absent and FVIII:C levels are usually around 1.0–2.0 iu/dL. These patients have a bleeding tendency that also has features of severe haemophilia A, with haemarthroses, muscle bleeds and life-threatening haemorrhage after trauma as well as a propensity to small-vessel bleeding (due to lack of VWF adhesive function), which is not a feature of haemophilia A.

Mild bleeding symptoms of the type typical of VWD are very common in the general population and thus have a low specificity for the presence of a bleeding disorder. It is now recommended that a simplified bleeding score (e.g. the international Bleeding Assessment Tool: BAT) is used to assess the likelihood of a significant defect in haemostasis before referring for investigation and that the result is borne in mind when interpreting the results of laboratory analyses. It will have greatest value when administered early in consultation, for example in primary care.

Laboratory diagnosis

Preliminary diagnosis

After obtaining a suggestive personal history and a family history, the laboratory diagnosis of VWD rests on assessing both the amount of VWF present (VWF:Ag) and its functional capacity. VWF:Ag is measured using immunological techniques; either an ELISA or an immunoturbidimetric assay. At present, it is generally possible to assess three important functions of VWF:

1. Platelet-dependent function: This function describes the ability of VWF to bind to and agglutinate platelets. It depends on the presence of high-molecular-weight VWF multimers and a functional binding site for platelet GPIb. Originally, dilutions of patient plasma were tested for their ability to promote platelet agglutination in the presence of the antibiotic ristocetin which serves to unfold the VWF molecule and mimic the effect of shear flow. This function was called ristocetin cofactor activity (VWF:RCo). A number of modifications now allow this to be performed on an automated analyser, using lyophilised platelets or recombinant GPIb bound to latex or magnetic particles (VWF:GPIbR). A further development utilises a mutant GPIb molecule that does not require ristocetin to interact with VWF (VWF:GPIbM). This avoids interference from naturally occurring variants that alter ristocetin binding but not platelet GpIb binding. Finally, an antibody to the A1 domain has been used to assess platelet binding activity (VWF:Ab). Automation has greatly improved the precision of these assays. Table 41.4

2. Collagen-binding function (VWF:CB): This is performed in an ELISA-based assay in which a well coated with collagen is used to capture VWF. It is dependent on the presence of high molecular weight multimers, as well as the functional binding sites for collagen on the A3 and A1 domains.

Thus, both VWF:RCo and VWF:CB are sensitive to the loss of high-molecular-weight multimers but measure different binding properties of VWF. They should be seen as complementary rather than alternative assays.

3. FVIII binding (VWF:FVIIIB): Assessed first by a FVIII assay and then if this is reduced, by an assay of VWF FVIII-binding capacity using a modified ELISA-based technique.

Secondary tests for classification of VWD

If a deficiency suggestive of VWD is detected then further tests, particularly multimer analysis (by electrophoresis) and ristocetin-induced platelet aggregation (RIPA) should be performed to allow accurate classification. (Figure 41.7)

Table 41.4 Assays or von Willebrand factor.

Measure	Definition	Method
VWF:Ag	Immunologically detected protein	ELISA or immunoturbidimetric assay
VWF:RCo	Ristocetin-induced platelet aggregation	Platelet aggregometry Automated analyser turbidimetric assay with modifications applied.
VWF:GPIbR	Aggregation of particles coated with recombinant GPIb in presence of ristocetin.	Immunoturbidimetric or Chemoluminescent
VWF:GPIbM	Aggregation of particles coated with a gain of function recombinant GPIb (ristocetin not required)	Immunoturbidimetric
VWF:Ab	Binding of antibody to VWF A1 domain	Immunoturbidimetric
VWF:CB	VWF binding to collagen	ELISA. Plate usually coated with mixture of type I/type III collagen.
VWF:FVIIIB	Factor VIII binding to VWF	ELISA. Plate coated with VWF and bound FVIII detected by antibody or chromogenic assay

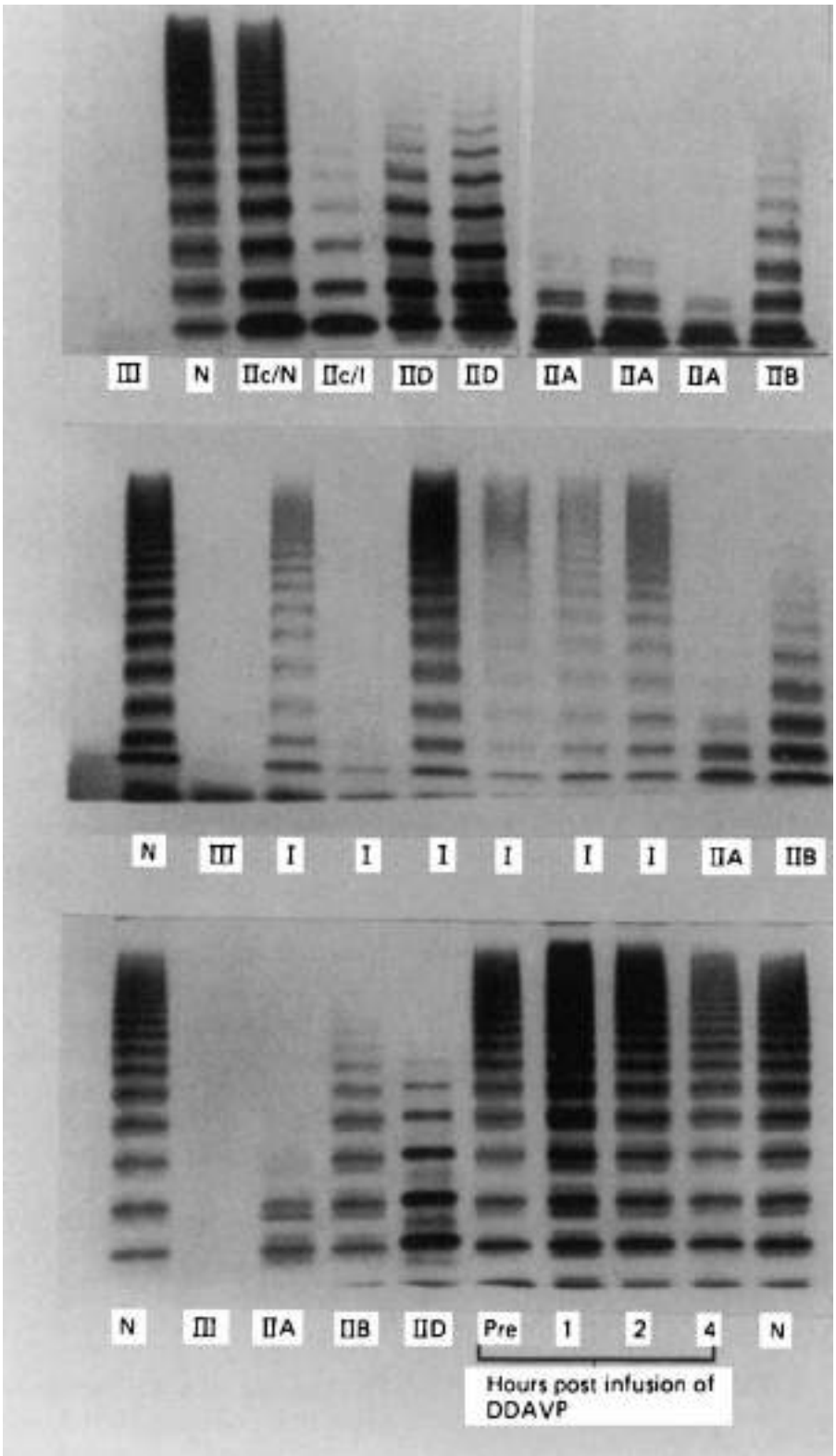

Figure 41.7 Multimer analysis of VWF from patients with VWD and normal control subjects. Note that many cases classified at present as type 2A were previously subdivided according to the details of the abnormality of triplet pattern.

Table 41.5 Classification of von Willebrand disease.

Type	Description	Comments	Inheritance
1	Partial quantitative deficiency of VWF	Requires a function:antigen ratio >0.6 or >0.7 according to various guidelines.	Mostly autosomal dominant inheritance when VWF <0.3 IU/mL. Levels >0.3 IU/ml may not segregate with *VWF* and associated variants show variable penetrance
1C	Quantitative deficiency (type 1) arising from accelerated clearance	Defined by >30% fall from peak at 4 hours post desmopressin or propeptide:antigen ratio >3	Archetype is VWF Vicenza due to the variant R1205H: inherited as autosomal dominant.
2	Qualitative VWF defects		
2A	Decreased VWF-dependent platelet adhesion with selective deficiency of high-molecular-weight multimers	Some controversy exists regarding classification of VWF mutations associated with subtle reductions in HMW multimers	Mostly autosomal dominant
2B	Increased affinity for platelet GPIb	Should be distinguished from platelet type pseudo-VWD (PT-VWD), using either platelet agglutination tests or genetic testing. Cases with normal VWF multimer and platelet count have been described	Autosomal dominant
2M	Decreased VWF-dependent platelet adhesion without selective deficiency of HMW multimers	This also includes defects of VWF collagen binding. May be combined quantitative/qualitative defect	Autosomal dominant
2N	Markedly decreased binding affinity for FVIII	Should be distinguished from mild haemophilia A	Reduced VWF:FVIII binding defects are frequently identified in a compound heterozygote state with a VWF null allele rather than the classical homozygous form
3	Virtually complete deficiency of VWF	Equivalent to <0.03 iu/mL in most antigen assays	Autosomal recessive, frequent null VWF alleles. Bleeding symptoms in 26–48% of obligate carriers

(*Source:* Modified from Laffan *et al.* (2014),)

(Table 41.5). The classification of VWD has been examined many times and extensive guidelines are available, detailed in the bibliography. What follows is necessarily a limited summary of much work.

Type 1 VWD is defined as a simple quantitative deficiency of VWF and the VWF present should be functionally normal with a normal multimeric pattern. In practice it is acceptable that the ratio of function to antigen (VWF:RCo/VWF:Ag) should be >0.7 and there should be no significant loss of high-molecular-weight multimers, even though on close inspection some subtle abnormalities of multimer pattern may be discerned. Some patients have a parallel reduction in antigen and function due to accelerated clearance of VWF from the circulation. This is now designated as type 1C and the archetypal variant is VWF Vicenza due to a R1205H change in VWF. Accelerated clearance of VWF can be detected by measuring an elevated ratio of VWF propeptide to VWF:Ag but is defined as a >30% fall from the post-desmopressin peak in 4 hours.

The characteristic of the 2A, 2B and 2M variants is a functional deficiency of VWF activity, which is reduced to below 0.7 of the antigenic measure. In type 2A, this results from a lack of high- and intermediate-size VWF multimers, whereas in type 2M a similar loss of platelet-binding activity is seen despite the presence of normal VWF multimeric composition and is caused by mutations in the *VWF* GPIb-binding site in A1. Loss of high-molecular-weight multimers in type 2A may arise from failure of intracellular assembly and processing or from accelerated proteolysis in the circulation due to mutations close to the ADAMTS-13 cleavage site. If multimer analysis is not available then a loss of high molecular weight multimers can be inferred from a reduction in both VWF:RCo and VWF:CB. VWD due to an isolated collagen-binding defect without loss of

high-molecular-weight multimers is rare, although a number of cases have been reported. The associated bleeding disorder is mild, perhaps because VWF contains two collagen-binding sites and the variants remain classified as 2M.

In type 2B, VWF binds prematurely to platelets in the circulation. *In vivo*, this results in the loss of high-molecular-weight multimers and platelets from the circulation due to formation of platelet aggregates and to increased cleavage of platelet-bound VWF by ADAMTS-13. The abnormality is detected in the laboratory by agglutination of platelets at a low concentration of ristocetin (0.5–0.7 mg/mL) that has no effect with normal VWF: the patient is then said to have positive ristocetin-induced platelet agglutination or RIPA. If normal washed platelets are resuspended in the patient's plasma, the phenomenon is reproduced, demonstrating that the abnormality is in the plasma. Thus RIPA should be performed as part of the diagnostic work-up for low VWF, as it is important to detect this variant (see below), which does not always show up in the other tests. Indeed a recent analysis showed that many patients with type 2B VWD have normal platelet counts and VWF multimer patterns. Most recent guidelines suggest that genetic analysis for variants in the A1 domain may be a preferred alternative to RIPA.

Type 2N VWD is characterised by VWF which has reduced affinity for FVIII (VWF:FVIIIB) but is normal in other respects. Preliminary investigations reveal only a reduced FVIII level (5–35%), which is easily mistaken for mild haemophilia. Inheritance is autosomal recessive which may provide a clue to diagnosis, but confirmation requires an assay of VWF FVIII-binding capacity or genetic analysis of FVIII and the VWF FVIII-binding site in the D′ domain.

Overall, the majority of patients are found to be type 1, which accounts for 75% of kindreds. Types 2A and 2B are fairly common, together accounting for about 15% of kindreds. Type 3 is the least frequent and has a prevalence of 1–2 in 10^6. Although classification has proved useful in understanding VWD and can help in planning treatment, some forms remain difficult to classify and may show features of more than one type.

Problems in diagnosis of type 1 VWD

The diagnosis of type 1 VWD implies that the patient has a significantly low level of VWF, which is responsible for an increased tendency to bleed. It is often not easy to be certain of this conclusion for the following reasons.

1. Slightly low levels of VWF are common in the population. Although the lower end of the normal range for VWF in the general population is approximately 50 iu/dL, most of the 2.5% of the population who have levels below this have normal haemostasis. In particular, individuals with blood group O have VWF levels on average 30% lower than those with non-O blood groups and the lower end of a 'blood group O normal range' is approximately 35 iu/dL. Thus the lower limit of the normal range is not the minimum level required for normal haemostasis. Nonetheless, individuals with blood group O are overrepresented in the group with type 1 VWD and there is a continuous relationship between bleeding tendency and VWF level that extends into the normal range without a clear cut-off between normal and abnormal. A firm diagnosis of VWD can be made when VWF function is <30 iu/dL but those with levels 30–50 iu/dL can be regarded as having 'low VWF'. This indicates that their VWF level is a risk factor for bleeding but will not always lead to a positive bleeding phenotype, most likely only when collagen and platelet function are insufficient to compensate.

2. A history of minor bleeding episodes (e.g. easy bruising and epistaxes) is very common in the population and is not a good predictor of more serious bleeding in other circumstances such as operations. On the other hand, a negative bleeding history may be because the patient has not yet have been exposed to a significant test of haemostasis.

3. As a result of the two points above, a history of, say, easy bruising and slightly low VWF levels will often be found together. However, this does not necessarily mean that the patient has VWD and caution should be exercised in drawing this conclusion as it has many consequences for the patient. Nonetheless, recent international guidelines have suggested that all patients in this group may benefit from being diagnosed as VWD largely on the basis that this will improve their access to treatment.

4. Intercurrent events such as stress, exercise, illness and pregnancy may all elevate the VWF level, making it difficult to be certain that a representative measure of VWF levels has been obtained from a single sample. It is, therefore, necessary to perform carefully standardised sets of assays and obtain concordant results on at least two occasions to be sure of the diagnosis and its severity.

5. The family history is often unhelpful, particularly in mild cases where penetrance is weak or variable. This is partly due to the modifying effects alluded to in (1) above and it is now evident that in some families, VWD does not segregate with the *VWF* gene. A large number of genetic variants in a wide variety of genes including *ABO* have been found to modulate VWF levels.

Treatment

Patients with mild or moderate VWD usually only need occasional treatment. Where possible, local control of bleeding should be used – such as the Mirena coil or combined oral contraceptive pill for HMB, referral to ENT for recurrent nose bleeds. The simplest general treatment is inhibition of fibrinolysis using tranexamic acid. Minor bleeding can sometimes be managed in this way and the addition of tranexamic acid mouthwash is often useful for oral cavity bleeding. Tranexamic acid is also frequently used in conjunction with desmopressin

which is the first choice specific treatment for minor bleeding after local measures have failed in type 1 VWD. Desmopressin will produce a brisk rise in VWF and FVIII levels (30 min after intravenous infusion) and a shortening of the bleeding time (for details of desmopressin therapy, see section on management of mild haemophilia A earlier in the chapter). Desmopressin is much less effective in types 2A and 2M, presumably because the patient's released VWF is abnormal and still unable to promote platelet adhesion. Almost all patients with a bleeding history and 'low VWF' will respond well to desmopressin providing there is not another contraindication. Desmopressin is generally regarded as contraindicated in type 2B VWD, as the released abnormal VWF will cause circulatory platelet aggregates to form, with a further fall in the platelet count. Before relying on desmopressin for therapy, patients should be given a test dose in order to determine the response at 30–60 min because this varies considerably. In addition, the duration of the response is also variable and further samples at 4–6 hours should be taken because some forms of type 1 VWD are characterised by a much-shortened survival of VWF (type 1C). A therapeutic trial may also be worthwhile in type 2A as some individuals do respond sufficiently for it to be useful. In type 2N, the FVIII response is of normal magnitude but is of limited efficacy due to its short duration, emphasising the importance of making the correct diagnosis, although this may still be useful for minor procedures.

In patients in whom desmopressin is ineffective or contraindicated, it is necessary to use a VWF-containing concentrate. Factor concentrates will always be required for treatment of type 3 VWD, most type 2 and those type 1 variants with inadequate desmopressin responses. Until recently all such concentrates were plasma-derived. Apart from the safety of the plasma source, the key feature of an effective concentrate is a full complement of high-molecular-weight multimers with a VWF:RCo/VWF:Ag ratio close to 1. Cryoprecipitate fulfils this requirement but cannot be subjected to viral inactivation procedures and is therefore not recommended as first-line therapy.

Most plasma-derived concentrates contain both FVIII and VWF in varying proportions. Because endogenous FVIII synthesis is normal, repeated treatment with these concentrates can result in high levels of FVIII which has been associated with thrombosis. Caution and monitoring are therefore required.

Recombinant VWF concentrate differs in some important respects. Firstly, it has not been exposed to plasma ADAMTS13 and contains ultra-large VWF multimers with correspondingly high specific activity. Secondly, it has a slightly different glycosylation profile and a longer half-life of survival in plasma. Thirdly, it does not contain any FVIII. When using either rVWF or high-purity plasma concentrates containing only VWF it is important to remember that following infusion there is a delay of approximately 6–12 hours before the level of FVIII rises substantially and, if rapid correction is required, FVIII concentrate should also be given.

Although many concentrates will not correct the bleeding time, this is not necessarily a bar to effective haemostasis. This seemingly paradoxical result may be because intra-platelet or extravascular VWF may be present. In situations when concentrates fail to stop bleeding, cryoprecipitate and platelet concentrates may prove effective.

Clinical course and complications

Patients with types 1 and 2 VWD lead relatively normal lives, with normal life expectancy. Menstruation is seldom a cause of severe blood loss, although HMB is common. This can usually be managed satisfactorily with hormonal therapy. If these are not effective, then additional tranexamic acid and even self-administration at home of desmopressin by intranasal or subcutaneous routes can be useful. Finally, intermittent prophylaxis with VWF concentrate can be employed. During pregnancy, the VWF levels rise spontaneously to the normal or low-normal range in most patients with type 1 VWD. In type 2 VWD, the response is unpredictable and should be closely monitored. Type 2B VWD is particularly complicated in pregnancy because the rise in endogenous VWF production exacerbates the thrombocytopenia. Patients with severe type 3 disease have a clinical course resembling severe or moderately severe haemophilia A, including the development of joint damage and should be considered for prophylaxis. Some patients with other forms of VWF will have recurrent bleeding and should also be considered for prophylaxis. A small number of patients with type 3 disease develop antibodies to VWF, which inhibit its platelet adhesion-promoting property and cause rapid removal from the circulation of infused material. Unlike anti-FVIII antibodies, some anti-VWF antibodies may mediate anaphylactic shock.

Molecular genetics

The cloning of VWF cDNA and its gene has led to progress in identifying the underlying mutations responsible for the various phenotypes. A database of mutations responsible for VWD has been established and curated at https://databases.lovd.nl/shared/genes/VWF

Type 1

There have been extensive studies to determine the molecular basis of type 1 VWD and a number of important points have emerged. Firstly, in about 35% of cases reported to have VWD, the disorder does not segregate with the VWF locus and is therefore presumably results from a combination of other modifying genes, in particular the ABO locus. However, this figure depends on the definition of type 1 VWD and the phenomenon is most common in individuals with VWF levels above 30 units/dL and includes a disproportionate number of blood group O individuals. The likelihood of the disorder segregating with the VWF gene and of a causative mutation being identified increases significantly as the VWF level falls and a mutation is found in all

cases where the VWF multimer pattern is abnormal. Amongst those with type 1 VWD and an identified mutation, mutations are widely scattered throughout the molecule.

Type 2

Mutations responsible for type 2 VWD affect a relevant functional site in the molecule and the corresponding portion of the gene. Mutations causing types 2M and 2B both affect the binding site for platelet GPIb on VWF and are found within the A1 domain of the molecule. They are inherited dominantly. As discussed above, type 2A may arise from increased susceptibility to proteolysis by ADAMTS-13 caused by mutations in the A2 domain. Otherwise, type 2A mutations are scattered throughout the gene but particularly in the propeptide, D′–D3 and the cysteine knot. In general, type 2A is dominant but occasional recessive forms occur. Mutations causing type 2N are found in the FVIII-binding site in the D′ domain but because the binding capacity of VWF greatly exceeds the FVIII concentration the inheritance is recessive.

Type 3

Type 3 is characterised by the complete absence of VWF and so arises from inactivating or null mutations in both VWF alleles and is inherited in an autosomal recessive manner. The rare patients who develop antibodies to VWF nearly all have large deletions of their VWF gene.

Pseudo von Willebrand disease (platelet-type)

Several families have been described with a disorder closely resembling type 2B VWD, but in whom mixing experiments show the defect to be in their platelets rather than their plasma. Patients with pseudo-VWD or platelet-type VWD (PT-VWD) have moderately reduced levels of VWF:Ag and platelets, with an enhanced response of their platelet-rich plasma to low levels of ristocetin (0.5 mg/mL). The addition of normal cryoprecipitate to their washed platelets causes spontaneous aggregation, whereas the reverse experiment is without effect (compare with type 2B). Missense mutations in platelet membrane GPIb, such that it spontaneously binds higher multimers of VWF, have been shown to be the underlying cause of this autosomal dominant mild bleeding syndrome. Treatment has not been extensively evaluated, but it should probably be with normal platelet concentrates, rather than desmopressin or cryoprecipitate. Thus this syndrome should be excluded by mixing tests as above or by genetic analysis before diagnosing type 2B VWD.

Acquired von Willebrand syndrome

In a number of circumstances, patients may acquire a deficiency of VWF function that is not inherited. The commonest association is with a paraprotein that binds to VWF and accelerates clearance, producing a type 2A or type 3 pattern. Severe aortic stenosis or extracorporeal circuits can cause sufficient shear stress on VWF to accelerate ADAMTS13 cleavage and cause an acquired von Willebrand syndrome that resolves immediately after valve replacement or release from the pump. A similar phenomenon occurs in myeloproliferative disease when thrombocytosis is present and excess binding of VWF to platelets promotes its cleavage by ADAMTS13. VWF synthesis is reduced in hypothyroidism (Table 41.6). Treatment of the underlying disorder will improve the syndrome. If urgent treatment is required, then replacement therapy may be necessary (taking into account accelerated clearance) and IgG paraproteins will usually respond to intravenous immunoglobulin.

Table 41.6 Disorders associated with acquired von Willebrand syndrome and mechanism responsible.

Disorder	Mechanism	Treatment
Hypothyroidism	Reduced synthesis	Correct thyroid status
Aortic stenosis	Shear stress and increased ADAMTS-13 cleavage	Replace aortic valve
Myeloproliferative	Adherence to platelets and increased ADAMTS-13 cleavage	Restore platelet count to normal
IgG paraprotein	Immune	IVIG, chemotherapy to reduce paraprotein
IgM paraprotein	Immune	Chemotherapy (IVIG ineffective)
Wilms tumour	Adsorption	Tumour removal if possible

IVIG, intravenous immunoglobulin.

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CHAPTER 42

Rare inherited coagulation disorders

42

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Introduction

The rare coagulation disorders (RCDs), including the inherited deficiencies of coagulation factors fibrinogen, prothrombin, factor (F)V, combined FV and FVIII, FVII, FX, FXI, FXIII and of multiple deficiency of vitamin-K-dependent factors, are usually transmitted in an autosomal recessive manner. The incidence of RCDs in the general population is therefore low, and homozygous or double heterozygous deficiencies vary from 1 in 500,000 for FVII deficiency to 1 in 2 million for prothrombin (FII) and FXIII deficiencies (Table 42.1). This incidence is also strongly influenced by ethnicity and is significantly increased by a high rate of consanguinity in the population.

The most recent evaluation of the distribution of the RCDs is available on the World Federation of Haemophilia website (WFH, http://www.wfh.org/) whose survey is aimed at collecting epidemiological data and at providing information to haemophilia organisations and treatment centres involved in reducing or preventing complications of bleeding. This survey, including the participation of about 125 countries worldwide, tripled the number of identified patients. Amongst these FVII and FXI deficiencies appear to be the most prevalent RCDs, representing 40 and 24% of the total number of affected patients, respectively, followed by fibrinogen (11%), FV and FX (8% and 6%), FXIII (7%) and combined FV and FVIII (2%) deficiencies, while the rarest disorder was FII deficiency with a prevalence of 2% (Figure 42.1).

Clinical symptoms

Clinical symptoms amongst patients with RCDs differ significantly from one disorder to another, and from one patient to another, affected with the same disorder. Significant bleeding manifestations usually show in homozygous or compound heterozygous patients, while heterozygous patients, with approximately half-normal levels of coagulation factors are usually asymptomatic. The most commonly reported symptoms are mucosal tract bleeding and excessive bleeding at the time of invasive procedures, delivery in women and circumcision in boys (where it is customary). Bleeds that endanger life, such as in the central nervous system (CNS), and gastrointestinal (GI) bleeding appear to be less frequent than in haemophilia. These symptoms are more frequent in FX deficiency. Particular care should be paid to women affected by RCDs as they may experience excessive monthly bleeding associated with menstruation. Indeed, heavy menstrual bleeding (HMB) is not the only gynaecological problem that women with RCDs are more likely to experience, they are also at risk of increased bleeding from conditions such as haemorrhagic ovarian cysts, endometriosis, endometrial hyperplasia, polyps and fibroids. Pregnancy and childbirth, two important stages in the life of a woman, pose particular clinical challenges in women with RCDs, since information about these issues is very scarce and limited to just a few case reports.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 42.1 General features of autosomal recessive deficiency of coagulation factors. Prevalence refers to the severe form of deficiencies due to homozygous or compound heterozygous mutations.

Deficiency	Estimated prevalence*	Gene (chromosome)
Fibrinogen	1 in 1 million	*FGA*, *FGB, FGG* (all on 4q28)
Prothrombin (Factor II)	1 in 2 million	*F2* (11p11–q12)
Factor V	1 in 1 million	*F5* (1q24.2)
Combined factor V and VIII	1 in 1 million	*LMAN1* (18q21.3–q22) *MCFD2* (2p21–p16.3)
Factor VII	1 in 500,000	*F7* (13q34)
Factor X	1 in 1 million	*F10* (13q34)
Factor XI	1 in 1 million	*F11* (4q35.2)
Factor XIII	1 in 2 million	*F13A1* (6p24–p25) *F13B* (1q31–q32.1)
Vitamin-K-dependent coagulation factors	Reported in <50 families	*GGCX* (2p12) *VKORC1* (16p11.2)

* Including dysfunctional proteins.

Classification

The rarity of the RCDs limits our knowledge of their natural history. It is now well known that every RCD can have several bleeding symptoms, ranging from minor post-trauma bleeding to the most severe episodes, which may appear at birth or later in life. In some deficiencies, the residual coagulant activity level is directly proportional to the risk of developing a haemorrhage, but this is not true for all types of deficiencies. This information has been finally made available, in recent years, thanks to publication in the scientific literature of registries and databases collecting data on different aspects of specific deficiencies. Amongst these, the European Network on Rare Bleeding Disorders (EN-RBD), based on a cross-sectional study using data from 489 patients involving 13 European treatment centres, for the first time evaluated the correlation between the coagulant plasma activity level and clinical bleeding severity in each RCD. Clinical bleeding episodes were classified into four categories of severity, based on the location and the potential clinical impact, as well as the trigger for bleeding (spontaneous, after trauma or drug-induced). By means of linear regression analysis, this study found a strong association between coagulant activity level and clinical bleeding severity for fibrinogen, combined FV + VIII, FX and FXIII deficiencies. A weak association was found in FV and FVII deficiencies, while coagulation-factor activity level of FXI could not predict clinical bleeding severity. From the same study, it was also clear that the minimum level to ensure complete absence of clinical symptoms is different for each disorder. This leads to the conclusion that RCDs should not be considered as a single class of disorders, but instead, studies should focus on the evaluation of specific aspects of each individual RCD (see Table 42.2).

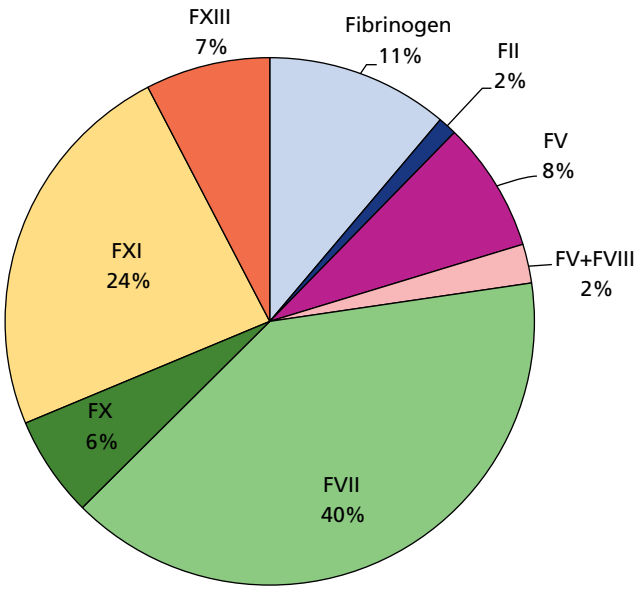

Figure 42.1 Worldwide distribution of rare coagulation disorders derived from World Federation of Haemophilia (WFH).

Laboratory diagnosis

The laboratory diagnosis of RCDs is currently carried out by means of coagulation screening tests, such as the activated partial thromboplastin time (aPTT) and the prothrombin time (PT), applied to subjects reporting a clinical and family history of bleeding. A prolonged aPTT with a normal PT is suggestive of FXI deficiency (after exclusion of FVIII, FIX and FXII deficiencies). The reverse pattern (normal aPTT and prolonged PT) is typical of FVII deficiency, whereas the prolongation of both tests directs further analysis towards possible deficiencies of FX, FV or prothrombin or to combined deficiencies. All coagulation tests that depend on the formation of fibrin as the endpoint will be affected by fibrinogen deficiency; hence, beside PT and aPTT, thrombin time (TT) should be performed. Abnormal results of screening tests should be followed by appropriate specific assays combined with mixing studies (50:50) to exclude the presence of a natural inhibitor of any deficient factor. Factor antigen assays are not strictly necessary for diagnosis and treatment but are can be used to distinguish type I from type II deficiencies; these measurements are very important in fibrinogen or FII deficiency, where normal antigen levels and reduced coagulant activity (dysfibrinogenaemia

Table 42.2 General recommendations for treatment and long-term prophylaxis in patients with inherited bleeding disorders.

	On-demand			Reported dose schedule for successful long-term prophylaxis			
Deficient factor (plasma half-life)	**Recommended trough levels**	**Product and dosages**	**Recommended trough levels (to maintain patient asymptomatic) after publication of the EN-RBD results**	**Products**	**Dose**	**Frequency**	**Notes**
Fibrinogen 2–4 d	0.5–1 g/L	Fibrinogen concentrate (50–100 mg/kg)	1 g/L	Fibrinogen concentrate	30–100 mg/kg	1/week	Afibrinogenaemic patients with recurrent life-threatening bleedings or undergoing surgeries. In afibrinogemic women, high level of fibrinogen is recommended to prevent early fetal loss (>1.0 g/L throughout pregnancy) and placental abruption during labour and to prevent PPH (ideally >2.0 g/L)
Prothrombin 3–4 d	20–30%	SD-treated plasma (15–20 mL/kg) PCC (20–30 units/kg)	>10%	PCC	25–40 units/kg	1/week	—
Factor V 36 h	10–20%	SD-treated plasma (15–20 mL/kg)	10%	SD-treated plasma	30 mL/kg	2/week	—
Factor V + VIII FV 36 h FVIII 10–14 h	10–15%	As for FV Mild FVIII: DDAVP Moderate and severe FVIII: pd- or rFVIII concentrate	40%	No data			—
Factor VII 4–6 h	10–15%	pdFVII concentrate (30–40 units/kg)	>20%	FFP	10–15 units/kg	2/week	Prevention of bleeding during surgery or in children with recurrent haemarthrosis or CNS bleeding
		PCC (20–30 units/kg)		pdFVII concentrate	10–50 units/kg	3/week	
		rFVIIa (15–30 µg/kg every 4–6 h)		rFVIIa	20–40 µg/kg	2–3/week	

(*Continued*)

Table 42.2 (*Continued*)

	On-demand			Reported dose schedule for successful long-term prophylaxis			
Deficient factor (plasma half-life)	**Recommended trough levels**	**Product and dosages**	**Recommended trough levels (to maintain patient asymptomatic) after publication of the EN-RBD results**	**Products**	**Dose**	**Frequency**	**Notes**
Factor X 40–60 h	10–20%	PCC (20–30 units/kg)		FX/FIX concentrate	20–40 units/kg	1–2/week	Patients with recurrent life-threatening bleeding or undergoing surgeries
		pdFX/FIX concentrate (10–20 units/kg)		Pd FX/FIX concentrate	20 units/kg	1/week	
		pdFX concentrate (25 units/kg)		pdFX concentrate	25 units	1/week	
Factor XI 50 h	15–20%	pdFXI concentrate (15–20 units/kg)	No data	Not indicated			—
Factor XIII 9–12 d	2–5%	pdFXIII concentrate (50 units/kg for high haemorrhagic events)		pdFXIII concentrate	10 units/kg	every 4–6 weeks	Highly recommended in severe patients
		rFXIII-A (35 units/kg)		rFXIII-A	35 units/kg	Every 4 weeks (2–3 in pregnancy)	
Vit.K-dep.		Vitamin K (10 mg i.v. or s.c. for minor bleeding)	No data available	Vitamin K	10 mg	1/week orally	—

d, days; h, hours; PCC, prothrombin complex concentrate; pdFVII, plasma-derived FVII; rFVIIa, recombinant activated FVII; SD, solvent/detergent.

and dysprothrombinaemia) can be associated with a risk of thrombosis. The standard laboratory clotting tests (PT, aPTT, fibrinogen level, platelet counts) are normal in FXIII deficiency. Its diagnosis can be detected by the demonstration of increased clot solubility in 5 M urea, dilute monochloroacetic acid or acetic acid. However, this method, qualitative and poorly standardised, detects only severe FXIII deficiency (with activity <5%), thus leading to a possible underdiagnosis. FXIII activity should be determined quantitatively by specific assays measuring ammonia released during the transglutaminase reaction or the incorporation of radioactive amines into proteins. In this case, a plasma blanking procedure is mandatory to avoid FXIIIa-independent ammonia release that can lead to incorrect results in the low-activity range below 5–10%.

Molecular diagnosis

Molecular diagnosis is based on the search for causative mutations in the genes encoding the corresponding coagulation factors. Exceptions are combined deficiency of FV and FVIII, caused by mutations in genes encoding proteins involved in FV and FVIII intracellular transport (*MCFD2* and *LMAN1*), and combined deficiency of vitamin-K-dependent proteins (FII, FVII, FIX and FX), caused by mutations in genes that encode enzymes involved in post-translational modification and in vitamin K metabolism (*GGCX* and *VKOR*). Due to the high allelic heterogeneity in RCDs, the best option is still to screen the entire gene, at least the coding portion and splice-site regions. Information on already identified mutations causing RCDs is easily traceable from the mutation database on the ISTH website (http://www.isth.org/MutationsRare Bleedin and also https://www.lovd.nl/). Missense mutations are the most frequent gene abnormalities, representing 50–80% of all identified mutations, except for *LMAN1* variants, where the most frequent mutations are insertions/deletions (50%). Insertion/deletion mutations represent 20–30% of the gene variations of the fibrinogen, *FV MCFD2* and *FXIII* genes and less than 15% of the remaining coagulation-factor gene mutations. Splicing and nonsense mutations comprise 5–15% of all identified mutations in all coagulation factors. Variants located in the 3′ and 5′ untranslated regions of the genes are the least frequent types of mutation (less than 5%). Despite significant advances in our knowledge of the genetic basis of the RCDs, the 'traditional' and standardised methods such as the Sanger sequencing and the Multiplex Ligation-dependent Probe Amplification (MLPA) fail to detect a genetic defect in 5–10% of patients affected with severe clotting-factor deficiencies. In these patients, the use of next-generation sequencing (NGS) might help to identify non-coding variants, novel genes and novel pathways in coagulation disorders. NGS platforms sequence millions of small fragments of DNA which need bioinformatic analysis to map the individual reads to a human reference genome. Each of the three billion bases in the human genome is sequenced multiple times, providing high depth to deliver accurate data and an insight into unexpected DNA variation. The explosion in data requires careful examination to determine the relationship with phenotype without assuming that every variant is pathogenic. Early work conducted in mouse models showed that complete absence of almost all coagulation factors led to a severe clinical phenotype often incompatible with life.

Global haemostasis tests

Tests investigating global haemostatic capacity, such as the thrombin generation test (TG) and thromboelastography (TEG) are of interest because standard coagulation tests, such as PT or aPTT have only limited sensitivity to reduction in coagulation factor levels. Therefore, global haemostasis tests may provide a more accurate evaluation of an individual's overall *in vivo* haemostatic state and response to treatment. They have been used to evaluate haemostasis in patients with RCDs and haemophilia and are now becoming a new laboratory strategy to determine the effectiveness of therapies and to monitor treatment in various bleeding situations in RCDs, in particular in those deficiencies, such as FXI, where standard assays fail to determine the bleeding risk.

Treatment

Treatment of RCDs is a difficult task, since information on management is often scarce, and replacement therapy of coagulation factors may require the prescription of unlicensed products that are not readily available. The main treatments are represented by non-transfusion adjuvant therapies (antifibrinolytic amino acids, oestrogen/progestogen) and replacement therapy for the deficient coagulation factor. Unfortunately, information on the safety and efficacy of the few available products in RCDs is sparse, and experience in their optimal use is significantly more limited than that available for haemophilia. The avoidance of transmission of blood-borne infectious agents is the primary requisite in the choice of replacement material. Solvent/detergent-treated plasma is an important source of replacement that is an option for the majority of these disorders. However, plasma-derived virus-inactivated concentrates or recombinant products should be preferred, when commercially available. Plasma should not be used when there are available concentrates since if repeated infusions are needed, as may occur during surgery or CNS bleeding, it may cause fluid overload. An updated online registry of the available clotting factor concentrates is published by the WFH (https://elearning.wfh.org/resource/online-cfc-registry/). A patient's personal and family history of bleeding are important guides for management. The conventional treatment for RCDs is given on an 'on demand' basis and is administered as

soon as possible after onset of bleeding; however, in clinically severe cases, a prophylactic regimen should be considered, as in afibrinogenemia and severe case of FVII and FX deficiency. Dosages and frequency of treatment depend on the minimal haemostatic level of the deficient factor, its plasma half-life, and the type of bleeding episode. The choice of prophylaxis is determined by the frequency of bleeding, the risk of severe spontaneous bleeding, and the risk of long-term disabilities associated with the occurrence of bleeding, despite on-demand treatment. General recommendations for treatment on demand and prophylaxis are summarised in Table 42.2.

Fibrinogen deficiency

Fibrinogen deficiency is heterogeneous and two main phenotypes can be distinguished: plasma levels of the protein are either not measurable or very low, leading to afibrinogenaemia and hypofibrinogenaemia. In other cases, low clottable fibrinogen contrasts with normal or moderately reduced fibrinogen antigen leading to dysfibrinogenaemia and hypodysfibrinogenaemia. As mentioned above, the laboratory determination of TT is a useful screening test in addition to PT and aPTT. However, the molecular defect may also have an impact on the results of the coagulation factor assays. Fibrinogen disorders have a heterogeneous genetic background. Fibrinogen is produced in the hepatocyte from three homologous polypeptide chains, Aα, Bβ and γ, which assemble to form a 340-kDa hexamer. The three genes encoding fibrinogen Bβ (*FGB*), Aα (*FGA*) and γ (*FGG*), ordered from centromere to telomere, are clustered in a region of approximately 50 kb on chromosome 4 in humans. Patients with afibrinogenemia are homozygotes or compound heterozygotes, while individuals with hypofibrinogenemia are usually heterozygotes. Dysfibrinogenemia is usually associated with autosomal dominant inheritance with two hotspots described, mutations located at *FGA p.Arg35* and *FGG p.Arg301*. These mutations are usually clinically benign, however, there are several other mutations that are associated with bleeding or thrombosis and predictive for the clinical phenotype, making genetic diagnosis beneficial where performance of TT is not part of routine coagulation.

Dysfibrinogenaemic and hypofibrinogenaemic patients are usually asymptomatic, while those with afibrinogenaemia have a bleeding tendency that usually manifests in the neonatal period, with 85% of cases presenting umbilical cord bleeding. Bleeding may also occur in the skin, gastrointestinal tract (GI), genitourinary tract or the CNS, while persistent damage to the musculoskeletal system and resulting handicap is less frequent. A milder symptom such as epistaxis is also frequent. Women may suffer from menometrorrhagia, but some have normal menses. First-trimester abortion is common in women with afibrinogenaemia, but less common in those with dysfibrinogenaemia. The conventional treatment is episodic and on-demand, but effective long-term secondary prophylaxis with administration of fibrinogen every 7–14 days has been described after CNS bleeds. However, high-level replacement, e.g. during pregnancy and the puerperium, should be moderated by the fact that thromboembolic events can occur.

The treatment of choice should be fibrinogen concentrate in case of afibrinogenemia and prophylaxis needs to be considered in those with a history of bleeding and for prevention of recurrent abortions in women.

Prothrombin deficiency

FII, a vitamin-K-dependent glycoprotein synthesised by the liver, is the zymogen of the serine protease α-thrombin and is encoded by a gene of approximately 21 kb located on chromosome 11. Prothrombin deficiency is one of the rarest inherited coagulation disorders, with a prevalence of about 1 in 2 million. As with fibrinogen deficiency, two main phenotypes can be distinguished: hypoprothrombinaemia (both activity and antigen levels are low) and dysprothrombinaemia (normal synthesis of a dysfunctional protein); hypoprothrombinaemia associated with dysprothrombinaemia was also described in compound heterozygosis.

Severe deficiency of FII, a condition in which plasma levels are <5% (homozygous or double heterozygous patients), is always characterised by severe bleeding, including prolonged post-injury bleeding, mucosal bleeding and subcutaneous and muscle haematoma. Conversely, GI bleeding episodes were reported in only a few cases. Dysprothrombinaemia manifests as a variable bleeding tendency that is usually less severe than true deficiency, while heterozygous subjects are usually asymptomatic although, occasionally, excessive bleeding after surgical procedures can be observed. In homozygous women, menorrhagia is frequent.

Replacement therapy is needed only in severe patients, in case of bleeding or to ensure adequate prophylaxis before surgical procedures. Since no FII concentrate exists, prothrombin complex concentrate (PCC) can be used for treatment. However, most PCCs contain other vitamin-K-dependent coagulation factors, which could potentially induce thrombotic complications. The novel non replacement therapies down-regulating natural anticoagulants might be a good therapeutic choice in the future.

Factor V deficiency

FV has a double role in the coagulation process: it is a protein cofactor required by the prothrombinase complex for thrombin generation but also contributes to the anticoagulant pathways by helping downregulation of FVIII activity and

supporting the survival of TFPI in plasma. The majority of individuals with FV deficiency are characterised by concomitant deficiency of FV activity and antigen levels (type I deficiency), but approximately 25% have normal antigen levels, indicating the presence of a dysfunctional protein (type II deficiency). FV is mainly secreted by hepatocytes but there is some evidence that it can also be synthesised *in vivo* in the megakaryocyte/platelet lineage. FV protein is encoded by a large (80 kb) and complex (25 exons) gene located on chromosome 1. Severe deficiency typically presents early in life, nonetheless, it is clinically heterogeneous, and sometimes, despite severe deficiency, patients may not bleed as much as expected. This has been attributed in part to the concomitant reduction in TFPI which circulates bound to the short splice variant form of FV. Observations made in the past few years point to an important role for platelet FV that provides new insight into this discrepancy. Megakaryocytes can synthesise FV; however, the majority of platelet FV is endocytosed from the plasma pool by megakaryocytes. Following endocytosis, FV is modified intracellularly; these changes to platelet FV appear to provide the cofactor with unique physical and functional characteristics, which render it more procoagulant compared with its plasma counterpart. Platelet degranulation and release of platelet FV at the site of vascular injury is thought to be a critical contributor to the local FV concentration. Furthermore, there is some evidence that, because platelet FV is locally released in high concentrations, it is less susceptible to inhibition and may function normally in haemostasis.

Noteworthy, loss-of-function mutations leading to FV deficiency are usually associated with a haemorrhagic disorder.

Symptomatic patients usually present with umbilical stump bleeding, skin and mucosal tract haemorrhages; epistaxis and menorrhagia are relatively frequent, even in patients with measurable FV levels. Postoperative and oral cavity haemorrhages are common, but not fully predictable, as these symptoms also occur with plasma levels as high as 5–10%. Haemarthroses and haematomas occur in only 25% of patients, and life-threatening bleeding episodes (GI and CNS bleedings) are rare. In patients with mild to moderate deficiency, therapy with antifibrinolytic drugs is sufficient to control epistaxis, menorrhagia or other non-life-threatening mucosal bleedings. Menorrhagia can also be managed directly using oral contraceptives, progestin-containing intrauterine devices, endometrial ablation or hysterectomy. Replacement therapy with FV has still to be administered as FFP, preferably virus inactivated, because no FV concentrate is yet available. A FV concentrate has been recently developed for clinical use in deficient patients and it has been shown to correct laboratory parameters (PT, PTT and thrombin generation) in severe FV deficiency plasmas. Unfortunately, this FV did not become available for the therapeutic use in FV deficiency. It has also been suggested that in cases of severe bleeding not controlled with FFP replacement, or in case of inhibitor development, platelet transfusions may be considered. A case of severe FV deficiency associated with multiple episodes of intracranial bleeding at birth was reported, where the inhibitor development owing to FFP infusion was managed by additional administration of platelets or rituximab.

Combined deficiency of factor V and factor VIII

Combined FV and FVIII deficiency (F5F8D) is characterised by concomitantly low levels (usually 5–20%) of the two coagulation factors, both as coagulant activity and as antigen. It appears that the concomitant presence of two coagulation defects does not enhance the haemorrhagic tendency observed in each defect separately. In 1988, F5F8D was shown to be causally associated with mutations in the *LMAN1* gene, encoding lectin mannose-binding protein (previously named *ERGIC-53*), a 53-kDa type 1 transmembrane protein that acts as a chaperone in the intracellular transport of both factors. In 2003, another locus associated with the deficiency was identified in about 15% of affected families, without mutations in *LMAN1*. This was named the *MCFD2* gene, encoding multiple coagulation factor deficiency (MCFD)2 protein, which acts as a cofactor for *LMAN1*, specifically recruiting correctly folded FV and FVIII in the endoplasmic reticulum. Further studies have failed to identify additional components of the *LMAN1::MCFD2* receptor complex, supporting the idea that causes of F5F8D might be limited to *LMAN1* and *MCFD2*. The phenotypes associated with mutations in *MCFD2* and *LMAN1* are indistinguishable and manifested only by deficiencies of FV and FVIII, although a selective delay in secretion of the cargo protein procathepsin C has been observed in HeLa cells over-expressing a dominant-negative form of *LMAN1*.

Recent studies, using thrombin-generation assays, showed that low levels of FV are accompanied by low TFPI levels thus supporting sufficient thrombin generation for optimal procoagulant activity.

Symptoms are usually mild, with a predominance of easy bruising, epistaxis and bleeding after dental extractions. Menorrhagia and postpartum bleeding have also been reported in affected women. More severe symptoms, such as haemarthroses and umbilical cord bleeding, are observed very seldom and GI and CNS bleeding have been reported in only a few patients. Soft-tissue haematomas are unusual. Because of the mild clinical pattern, bleeding episodes are usually treated on demand and do not require regular prophylaxis. These cases can be treated with desmopressin, as the low FVIII is usually of more significance than the low FV. More severe cases may need to be treated with FVIII concentrate and/or FFP as sources of both FV and FVIII may be needed when their different plasma half-lives (FV 36 hours, FVIII 10–14 hours) have to be taken into account.

Factor VII deficiency

FVII deficiency is the most common autosomal recessive coagulation disorder (1 in 500,000). A typical feature of this disease is its clinical heterogeneity, which ranges in severity from lethal to mild, or even asymptomatic forms. FVII is synthesised in the liver and is encoded by the FVII gene (*F7*) located on chromosome 13, 2.8 kb upstream of the *FX* gene. Plasma levels of FVII coagulant activity (FVII:C) and FVII antigen (FVII:Ag) are influenced by a number of genetic and environmental factors (sex, age, cholesterol and triglyceride levels) and it is also well known that FVII levels are modulated by *F7* polymorphisms. The majority of patients have concomitantly low levels of FVII:C and FVII:Ag, but several cases are characterised by normal or low borderline levels of FVII:Ag, contrasting with lower levels of FVII:C.

The severity of symptoms of FVII deficiency is variable: some patients do not bleed even with a very low level of FVII:C, while others with similar levels of coagulant activity have frequent bleeding. Life- or limb-endangering bleeding manifestations are relatively rare, the most frequent symptoms being epistaxis and menorrhagia. However, CNS bleeding was also reported to have high incidence (16%) in a series of 75 patients, the study concluded that the greatest risk factor for development of bleeding was trauma related to the birth process. Thrombotic episodes have also been reported in 3–4% of patients with FVII deficiency, particularly in the presence of surgery and replacement treatment, but spontaneous thrombosis may also occur; therefore, it can be inferred that patients affected with FVII deficiency are not protected from thrombosis.

A number of therapeutic options can be offered to patients with FVII deficiency, including FFP and PCCs (however, concentrations of FVII in FFP and PCC are low and therefore these are not the best treatments), plasma-derived FVII concentrates and recombinant FVIIa, which has to be considered the optimal replacement therapy because it can be used at very low dose (10–20 μg/kg) and is free from infection risk. Prophylaxis has been a debated issue in FVII deficiency, particularly in those patients with a severe bleeding history. However, the short half-life of infused FVII makes it difficult to establish a standardised protocol for prophylaxis. To improve rFVIIa half-life, longer-acting rFVIIa molecules have been generated by fusion protein or conjugation technologies: a rFVIIa molecule fused to albumin (rVIIa-FP) showed prolongation of half-life but is not yet in clinical use.

The occurrence of frequent menorrhagic symptoms is almost invariably associated with chronic anaemia associated with iron deficiency in women with the severe form of FVII deficiency. Pregnancy itself does not require special precautions and uncomplicated delivery is possible without prophylaxis. However, all cases of postpartum bleeding reported so far occurred in women with low FVII coagulant activity (<15 units/dL) not receiving prophylaxis. Therefore, delivery should take place with the protection of short-term replacement.

Factor X deficiency

FX is a glycoprotein that plays a pivotal role in the coagulation cascade, being the first enzyme in the common pathway of thrombin formation. FX is mainly synthesised by the liver and is encoded by the *FX* gene (*F10*), comprising 22 kb and located on chromosome 13, a few kilobases downstream of *F7*. FX deficiency may occur with concordant reduction in coagulant activity and antigen, or low coagulant activity contrasting with normal or low borderline antigen values.

In FX deficiency, the bleeding tendency may appear at any age, although the more severely affected patients (FX coagulant activity <1%) present early in life with umbilical stump, CNS or GI bleeding. Patients with severe deficiencies also commonly experience haemarthroses and haematomas. The most common bleeding symptoms reported at all levels of severity are epistaxis and menorrhagia.

Data from the UK Haemophilia Centre Doctors' Organisation (UKHCDO) registry shows that the proportion of patients with this deficiency who require treatment is much higher than that of other rare coagulation deficiencies. Heterozygous patients have also been reported to have bleeding after delivery that required treatment. Freeze-dried human coagulation FIX and FX concentrate with specified amounts of FX (and FIX) has facilitated prophylaxis in patients with FX deficiency and was developed in response to concerns about variability in coagulation factors in PCCs. Clinical trials investigating the pharmacokinetics of a high-purity plasma-derived FX concentrate have been also completed (ClinicalTrials.gov identifiers: 00930176, 01721681, and 01086852). An analysis of the clinical trial results together with post-marketing data showed only nonserious adverse reactions, with no inhibitor development and no thromboembolic events, resulting in a good safety profile of pdFX.

Therapeutic options for the control of menorrhagia are both medical (e.g. antifibrinolytics, oral contraceptives, levonorgestrel intrauterine device and clotting factor replacement) and surgical (e.g. endometrial ablation and hysterectomy). Although FX plasma concentrations increase slightly in pregnancy, women with the severe form of the deficiency and a history of adverse pregnancy outcomes (such as abortion, placental abruption or premature birth), may benefit from continuous replacement therapy.

Factor XI deficiency

FXI deficiency is characterised by a decrease in the functional activity of this plasma protein, usually accompanied by correspondingly low FXI antigen levels. More rarely, normal or borderline levels of plasma FXI antigen are associated with a dysfunctional form of the protein. The estimated prevalence of the severe FXI deficiency in most populations is about 1 in

1 million, but is reported to be much higher in Ashkenazi Jews, heterozygosity being as high as 8%.

FXI is mainly synthesised in the liver, although tiny amounts of transcript can also be detected in megakaryocytes and platelets. The protein is encoded by the *FXI* gene, comprising 23 kb and located on chromosome 4. At variance with other RCDs, whose pattern of inheritance is autosomal recessive, in some cases of *FXI*, missense mutations were shown to exert a dominant negative effect through heterodimer formation between the mutant and wild-type polypeptides, resulting in a pattern of dominant transmission. The existence of a platelet FXI transcript, originating from the skipping of exon 5, was hypothesised for a long time, but never confirmed by subsequent work. Nonetheless, data support the view that FXI transcripts undergo alternative splicing, leading to the synthesis of FXI isoforms whose physiological role and importance still need to be demonstrated.

The relationship between FXI levels in plasma and the bleeding tendency is not as clear-cut as for other coagulation factor deficiencies as also shown by the EN-RBD results. Usually, patients with severe FXI deficiency (1% or less) are mildly affected and most bleeding manifestations are injury-related. The phenotype of bleeding is not correlated with the genotype, but when a site of injury with local high fibrinolytic activity is involved (e.g. urogenital tract, oral cavity after dental extraction or tonsillectomy), the risk of bleeding is increased (49–67%) in comparison to sites with less local fibrinolytic activity (1.5–40%). Surprisingly, patients with low, but detectable, levels of FXI (1–5%) are also mild bleeders, so that clinical phenotypes are not strikingly different in these two groups. The most frequent symptom is oral and postoperative bleeding, which occurs in more than 50% of patients. Women with FXI deficiency are prone to excessive bleeding during menstruation, but case series of women affected by severe FXI deficiency showed that 70% of pregnancies were uneventful, despite no prophylactic treatment.

Treatment is based on the use of antifibrinolytic agents, FFP and FXI concentrate. Recombinant FVIIa has been successfully used in surgeries, but care should be taken to reduce the risk of complications such as thrombotic events (especially with FXI concentrate), volume overload, allergic reactions and development of inhibitors. Future studies are needed to confirm the role of rFVIIa in surgery and FXI deficiency. Patients with a partial deficiency in FXI who have no bleeding history do not require prophylactic treatment.

Factor XIII deficiency

FXIII is a transglutaminase and functions to cross-link the α and γ fibrin chains. It also cross-links antiplasmin to fibrin overall resulting in a stronger clot with an increased resistance to fibrinolysis. The plasma factor consists of two catalytic A subunits (FXIII-A) and two carrier B subunits (FXIII-B). FXIII-A is synthesised in cells of bone marrow origin, while FXIII-B is produced in the liver. The corresponding genes are located on chromosomes 6 and 1. FXIII deficiency is, together with prothrombin deficiency, the rarest of the recessively transmitted coagulation factor deficiencies (1 in 2 million). In inherited FXIII deficiency, plasma levels of FXIII-A measured as functional activity or immunoreactive protein is usually extremely reduced, whereas the FXIII-B subunit is reduced, but always at measurable levels. Patients with FXIII-A deficiency have a bleeding tendency that is usually severe, particularly because of the early onset of life-threatening symptoms such as umbilical cord and CNS bleeding occurring in up to 80 and 30% of patients, respectively. Ecchymoses, haematomas and prolonged bleeding following trauma are also typical. Haemarthroses and intramuscular haematomas may appear unexpectedly, although less frequently. In women of reproductive age, miscarriage and intraperitoneal bleeding are often reported. All these symptoms usually lead to an early diagnosis, so that patients who survive are often treated prophylactically, starting early in life.

This approach to treatment is rendered simple and feasible by the fact that plasma levels of FXIII of 2–5% are sufficient to prevent severe bleeding and that the long *in vivo* half-life of the factor (11–14 days) makes it possible to infuse cryoprecipitate or concentrates at intervals of 1 month or longer. The recent prospective data collection project (PRO-RBDD; www.rbdd.org) showed that a level of 15% of FXIII clotting activity may represent a good therapeutic target to maintain patients without bleeding.

Plasma-derived FXIII has been used for several years and shown to be safe and effective; however, a recombinant FXIII-A_2 concentrate (rFXIII-A_2) has also become available Its approval was based on the evaluation in the mentor™ clinical trial programme (mentor™1, 2 and 5), in which 82 patients were monitored closely showing that monthly rFXIII-A2 prophylaxis has a good safety profile and provides a low bleeding rate in adults and children with FXIII-A deficiency. Results on the long-term safety and effectiveness of rFXIII-A2 prophylaxis in real-world use from a non-interventional post-authorisation safety study mentor™6 confirmed that it is well tolerated as long term care.

Only a few cases of inherited FXIII-B deficiency have been reported to date, and only 16 causative mutations have been identified; FXIII-B deficiency bleeding symptoms appear milder than in FXIII-A-deficient patients.

Vitamin-K-dependent coagulation factors deficiency

Vitamin-K-dependent coagulation factors such as FII, FVII, FIX and FX require γ-carboxylation of glutamic acid residues in their Gla domains to enable binding of calcium and attachment to phospholipid membranes. The γ-carboxylation is catalysed

by hepatic γ-glutamyl carboxylase (GGCX) and its cofactor, reduced vitamin K (KH_2). During the reaction, KH_2 is converted to vitamin K epoxide (KO), which is recycled to KH_2 by the vitamin K epoxide reductase (VKOR) enzyme complex. Heritable dysfunction of GGCX or the VKOR complex results in the secretion of poorly carboxylated vitamin-K-dependent coagulation factors, leading to combined deficiency of the vitamin-K-dependent clotting factors. The γ-carboxylation of glutamic acid residues is also required for activity of the anticoagulant factors protein C, protein S and protein Z, but although protein S and protein C levels are low in VKCFD, there are no reports of venous or arterial thrombosis. Thus, the effect of VKCFD is clearly towards bleeding. The GGCX and VKOR proteins are encoded by two corresponding genes: *GGCX* (13 kb, 15 exons) located on chromosome 2 and the unusually small *VKORC1* (5126 bp, 3 exons) located on chromosome 16; the latter gene was so named because of evidence suggesting that VKOR is a multisubunit complex.

VKCFD is a rare autosomal recessive bleeding disorder that often presents during infancy, although the routine administration of vitamin K may delay the diagnosis of VKCFD in neonates. Severe symptoms, such as intracranial haemorrhage or umbilical stump bleeding, are usually associated with factor levels below 5 units/dL. Severely affected children may also present with skeletal abnormalities such as nasal hypoplasia, distal digital hypoplasia, epiphyseal stippling and mild conductive hearing loss. Older patients can present with easy bruising and mucocutaneous or postsurgical bleeding.

Treatment with oral or parenteral vitamin K should be started as soon as possible in all patients at diagnosis. However, some patients show insufficient response, and there are limited data on the effectiveness of prophylaxis with weekly administration of vitamin K_1 10 mg. In fact, massive parenteral doses of vitamin K do not always correct FII, FVII, FIX and FX activities and there is clear biochemical evidence that the molecules are not fully carboxylated by such treatment. In this group of patients, factor replacement by virally inactivated FFP could also be used in cases of acute bleeding episodes or before surgery. PCC is the alternative choice when a rapid increase in clotting factor levels is necessary.

Concluding remarks

Rarity of RCDs limits the in-depth analysis of their clinical and laboratory features and limit their knowledge, in particular amongst general physicians, increasing the risk of misdiagnosis and fatal consequences. Therefore, the establishment of global networks of treatment centres is mandatory to improve the amount of collected data, to strengthen the power of the analysis deriving from such data, and to improve and standardise laboratory assay to define the minimum residual level of coagulation factor to ensure a normal haemostatic process. The resulting definition of the clinical endpoints will facilitate the design of appropriate clinical trials for the evaluation of the safety and efficacy of novel drugs. Multinational multicentre data collection for long-term post-registration surveillance remains an important milestone in the study of RCDs.

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CHAPTER 43

Acquired coagulation disorders

43

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Introduction

Acquired disorders of haemostasis are a heterogeneous group of conditions with varied and often complex aetiologies. Patients may have multiple and overlapping causes for bleeding and distinct conditions often have similar pathophysiologies. To manage acquired haemostatic failure it is important to understand the mechanisms by which haemostatic disturbance occurs and how these apply in different ways to a variety of conditions.

Systemic disease may present to haematologists with symptoms of bleeding or bruising or abnormalities of coagulation tests. Alternatively, patients with known disorders may need haematological input to manage symptoms or at the time of invasive procedures. Assessment of patients with symptoms of bleeding or bruising requires clinical review and laboratory investigation. Serial laboratory testing is often required because the haemostatic disturbance may evolve rapidly. The possibility of a congenital disorder and potential role of anticoagulant or antiplatelet drugs should be considered.

Haematologists are also often involved in the management of acutely bleeding patients who may have complex medical conditions and in whom bleeding is usually multifactorial. Critical to optimising the management of these patients is a well-organised multidisciplinary team approach. Hospitals should have systems in place to respond rapidly to massive haemorrhage and hospital transfusion committees should establish protocols for these medical emergencies. Laboratories should be geared to producing accelerated full blood count (FBC) and coagulation results and point-of-care tests of haemostasis may be useful. The rapid supply of red blood cells and components and efficient transfer of these to the patient is vital to success. 'Fire drills' to test responses to emergencies are good practice.

Tests of coagulation and point-of-care testing

Routine tests of haemostasis

To investigate a patient suspected of an acquired haemostatic defect, a platelet count is required and examination of the blood film is often useful. Routine coagulation tests include the prothrombin time (PT), activated partial thromboplastin time (APTT) and Clauss fibrinogen level. Derived fibrinogen assays should not be used because results are likely to be misleading.

If screening tests are abnormal, correction studies help to distinguish factor deficiencies from inhibitors. Comparison of TT with the reptilase time or direct measurement of heparin anti-Xa levels will establish whether abnormal results are due to heparin. Measurement of fibrin breakdown products, such as D-dimers, is required for investigation of possible disseminated intravascular coagulation (DIC). Measurement of individual coagulation factor and von Willebrand factor (VWF) levels may be required. Assessment of platelet function may be useful in some circumstances, through platelet aggregation studies, platelet nucleotides or a bleeding time, although these results may be difficult to interpret in the context of an acquired haemostatic defect. The role of global platelet function analysers such as PFA-100 remain to be defined but are available in many hospitals.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Thromboelastometry

Tests based on thromboelastometry are point-of-care tests that assess the viscoelastic properties of whole blood under low-shear conditions and their use is becoming increasingly common. Their main advantages are the assessment of the different components of clot formation, including platelets and coagulation factors and rapid availability of results. They are used to guide blood product administration in patients undergoing liver transplantation, cardiopulmonary bypass and at the time of traumatic or obstetric haemorrhage. The main instruments are TEG, ROTEM and Sonoclot, and these measure the viscoelastic properties of an evolving clot using distinct technologies. Results are displayed graphically and show the developing and dissolving clot, although current assays are relatively insensitive to fibrinolysis and cannot be used to routinely guide tranexamic use. Heparinase cups can be used to investigate the underlying haemostasis of heparinised samples. Surrogate assays that reflect the contribution of fibrinogen to haemostasis are available. If thromboelastometry is used in these circumstances, hospitals should have agreed algorithms for interpretation and consequent infusion of blood products and undertake internal and external quality control.

Thrombin generation assays

These tests measure the amount of thrombin generated over time and may reflect an individual's coagulation potential. Assays are initiated by the addition of a trigger, usually tissue factor (TF), to recalcified plasma in the presence of phospholipids. Thrombin is detected through cleavage of a chromogenic or fluorogenic substrate. When low concentrations of TF are used, contact activation must be inhibited by corn trypsin inhibitor. Several variations of these assays have been developed by varying the concentrations of TF, corn trypsin inhibitor, phospholipid and protein-C-sensitising reagents such as soluble thrombomodulin. The clinical utility of thrombin generation assays remains to be demonstrated and they are not routinely available.

Activated clotting time

This test is used to monitor anticoagulation with heparin during cardiopulmonary bypass (CPB) surgery. It uses whole blood and has a linear response at the high concentrations of heparin used during CPB, extracorporeal membrane oxygenation and percutaneous coronary intervention. The APTT cannot be used because the assay is insensitive to the high heparin concentrations. Measured using a specialised analyser, the activated clotting time (ACT) target range varies according to the method and usually falls within 70–180 s. During heparinisation for CPB, the usual goal is to achieve 400–500 s. Off-pump cardiac surgery has been described using less anticoagulation and lower ACT target ranges of 200–300 s.

Disseminated intravascular coagulation

Background

DIC is characterised by the loss of localisation or compensated control of intravascular activation of coagulation. Arising from diverse causes (Table 43.1), its pathology can manifest systemically and contribute to a worse prognosis for the patient. Uncoupling of the highly regulated balance between procoagulant, anticoagulant, profibrinolytic and antifibrinolytic processes can result in simultaneous bleeding and microvascular thrombosis at different vascular sites (Figure 43.1). Removal of the inciting cause is the best means of restoring haemostatic control but may not be possible. Sepsis and trauma cause more than half the cases of DIC.

Table 43.1 Clinical conditions associated with disseminated intravascular coagulation.

Sepsis/severe infection
Potentially any microorganism, including Gram-positive and Gram-negative bacteria, viruses, fungi and rickettsial infections
Malaria and other protozoal infections
Trauma
Serious tissue injury
Head injury
Fat embolism
Burns
Malignancy
Solid tumours
Haematological malignancies (e.g. acute promyelocytic leukaemia)
Obstetric complications
Placental abruption
Amniotic fluid embolism
Pre-eclampsia
Intrauterine fetal demise
Vascular abnormalities
Giant haemangiomas (Kasabach–Merritt syndrome)
Large vessel aneurysms (e.g. aortic)
Severe toxic or immunological reactions
Snake bites
Recreational drugs
Severe transfusion reactions
Transplant rejection
Miscellaneous
Severe pancreatitis
Heat stroke
ABO mismatch transfusion

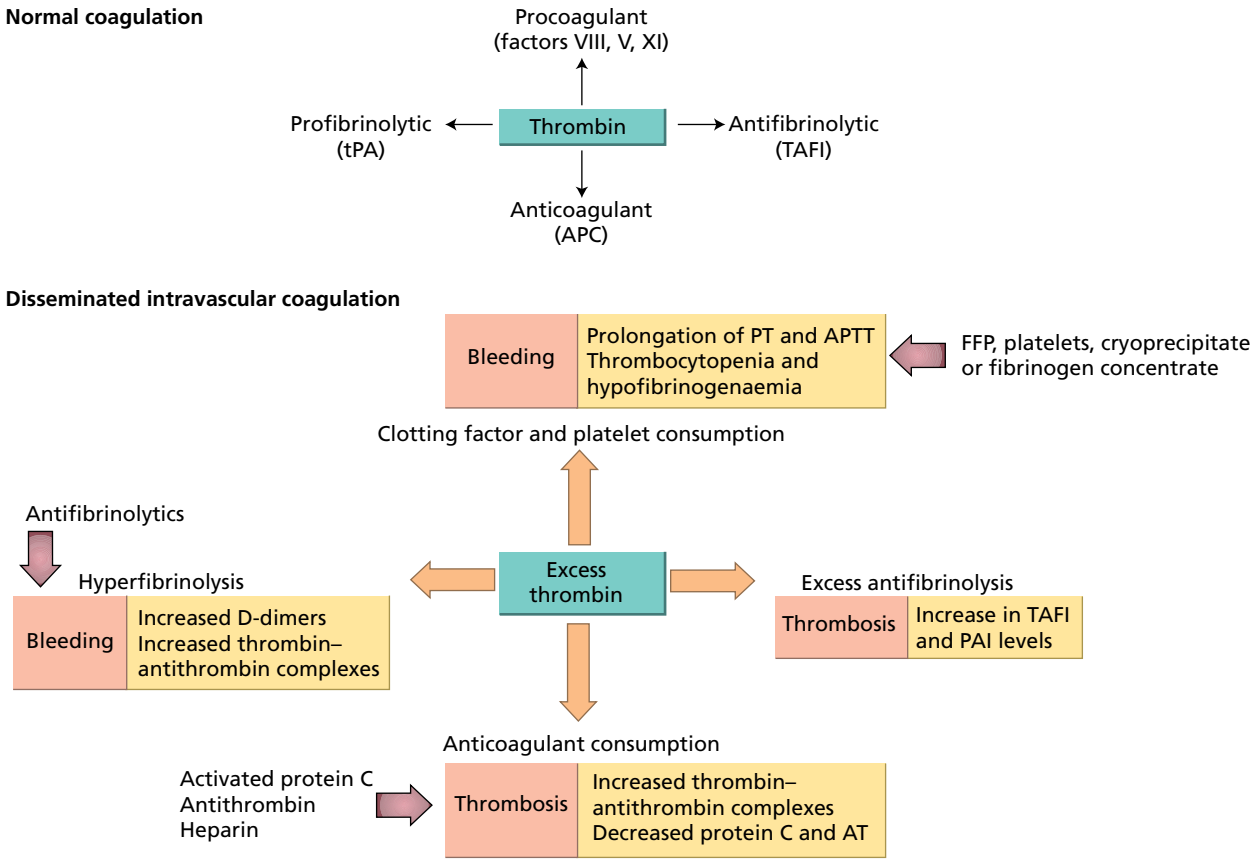

Figure 43.1 The changes in disseminated intravascular coagulation are compared with the normal coagulation. The excess thrombin generation in DIC leads to either bleeding or thrombosis (depicted in red boxes) based on the predominant coagulation change (shown in yellow boxes) that has occurred. The therapeutic intervention in each setting is given by the arrow towards each double box. AT, antithrombin; PAI, plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor.

Pathophysiology

There are several important themes in the pathophysiology of DIC (Figure 43.1): first is the central role played by the generation of thrombin; second, mechanisms that perpetuate thrombin generation allowing it to become pathogenic in its dissemination; third, parallel and concomitant activation of inflammation and fourth, the importance of the endothelial microvasculature in this process.

Excess bleeding in DIC is partly attributable to the depletion of coagulation factors and platelets. However, other factors may contribute, including abnormal platelet function and hyperfibrinolysis. An additional factor possibly is the widening of the endothelial gap junctions caused by inflammation due to the underlying disease and lack of protection for the endothelial integrity provided by the platelets. Conversely, microvascular thrombosis can be precipitated by reduced levels of circulating anticoagulant proteins as well as loss of cell surface receptors, such as thrombomodulin, and glycans from endothelium.

Thrombin generation *in vivo*

The TF pathway plays a major role in initiating thrombin generation. In health, TF is extravascular mainly present on fibroblasts and smooth muscle cells surrounding blood vessels In contrast, in sepsis, infecting micro-organisms induce TF expression on monocytes and other inflammatory cells, while in trauma tissue factor and phospholipid can be released from injured tissues such as the brain. During obstetric complications, the placenta and amniotic fluid act as rich sources of TF, while malignant cells release TF-bearing extracellular vesicles in the cancer setting. Although the TF pathway is considered more important in thrombin generation in DIC, the contact pathway through factor (F) XII activation contributes to the pathological state by activation of the kallikrein–kinin system, causing vasodilatation, hypotension and activation of fibrinolysis. In situations of severe sepsis and septic shock, these intrinsic pathway components become much more relevant and can contribute significantly to the morbidity and mortality of the patients.

Mechanisms for disseminating and sustaining thrombin generation

When the inciting insult is persistent or severe, the amount of thrombin generated becomes continuous and excessive. This can lead to consumption and depletion of coagulation and anticoagulant factors. Increased exposure of negatively charged phospholipid surfaces facilitates the assembly and enhances the rate of coagulation reactions. Such surfaces, rich in phosphatidylserine and phosphatidylethanolamine, are provided by externalisation of the inner leaflet of cell membranes upon activation and apoptosis. Cell damage also leads to the generation of extracellular vesicles from platelets, monocytes and endothelial cells that express tissue factor and increase the circulating surface area for coagulation reactions. Phospholipid surfaces are also provided by very low-density lipoprotein, which can increase several-fold in severe sepsis to further enhance and sustain thrombin generation. Polyphosphate released from activated platelets and invading microorganisms in sepsis can accelerate coagulation via the contact pathway and also inhibit tissue factor pathway inhibitor and delay clot lysis by stimulating thrombin-activatable fibrinolysis inhibitor. Together, these mechanisms promote a spatially and temporally expanded response that is the hallmark of DIC.

Links between inflammation and coagulation

Once activated, the inflammatory and coagulation pathways interact to amplify the response. Cytokines and proinflammatory mediators such as tumour necrosis factor (TNF), interleukin (IL)-1 and high mobility group box protein (HMGB)-1 induce activators of coagulation. Thrombin and other serine proteases interact with protease-activated receptors (PARs) on cell surfaces to promote inflammation. When this process escapes, the well-developed local checks, it results in a dysregulated response that fuels a vicious cycle. An important role in interactions between coagulation and the complement system has also been increasingly identified in DIC.

Endothelial cell activation and dysfunction

Dysfunction and failure of the endothelium can lead to the development or propagation of DIC. The degree to which this occurs and the dominance of thrombotic or bleeding sequelae depend on the cause of DIC and host-related factors. Damage to and activation of the endothelium downregulates and depletes its anticoagulant receptors, such as thrombomodulin and endothelial protein C receptor, exposes subendothelial collagen that binds activated platelets, and releases plasminogen activator inhibitor (PAI)-1, which inhibits fibrinolysis, and ultra-large VWF multimers, which increase platelet aggregation. Endothelial dysfunction can lead to the depletion of nitric oxide and resultant uninhibited platelet activation.

Neutrophil extracellular traps and histones

Neutrophils release extracellular traps (NETs), web-like structures with cytosolic and granular proteins assembled on chromatin, which aid in the removal of pathogens. NETs can activate coagulation by assisting in the extracellular delivery of tissue factor, degrade tissue factor pathway inhibitor, stimulate platelets causing aggregation, and cause endothelial damage. Thus, a beneficial antimicrobicidal function can become deleterious, causing disseminated coagulation. Further, high levels of histones have been detected in the plasma of experimental animals, and human patients with sepsis and trauma. Extracellular histones released after cell damage can cause massive thromboembolism associated with consumptive coagulopathy and are major mediators of death in sepsis. It can also cause acute lung injury in trauma models and may be a useful therapeutic target.

Clinical features

The perturbed coagulation associated with DIC can manifest clinically at any point in a spectrum varying from bleeding to thrombosis. Although bleeding, ranging from oozing at venepuncture sites to major gastrointestinal or intracranial haemorrhage, is the archetypal and most obvious manifestation of DIC, organ failure due to microvascular thrombosis is more common and often unrecognised. For example, in meningococcal septicaemia and rarely pneumococcal infection, thrombosis of the adrenal vessels can lead to adrenal insufficiency and Waterhouse–Friderichsen syndrome.

Diagnosis

DIC is a clinicopathological syndrome and, as such, there is no single laboratory test that can confirm or refute the diagnosis. In clinical practice, the diagnosis is usually made by a combination of routinely available coagulation tests in a clinical situation where DIC is suspected. DIC must be differentiated from other acquired disorders of haemostasis (Table 43.2). The typical findings are a prolonged PT and APTT (although may not be seen in 50% of cases with evidence of DIC), elevated products of fibrin breakdown (e.g. D-dimer), thrombocytopenia and falling fibrinogen. However, results within the normal range do not exclude a significant consumptive coagulopathy, because the acute-phase response shortens the APTT and increases fibrinogen. A fall in platelet number within the normal range may be significant.

It is important to recognise that DIC is a dynamic process and thus interpreting a series of laboratory tests over time is more relevant than looking at a single set of results. The ISTH Sub-Committee of the Scientific and Standardisation Committee on DIC has recommended the use of a scoring system for overt DIC (Table 43.3). This has been prospectively validated, indicating a very high sensitivity and specificity. A strong correlation

Table 43.2 Differential diagnosis of disseminated intravascular coagulation (DIC).

Condition	Similarities	Differences
Liver disease	Bleeding common PT, APTT abnormal Platelet count low Fibrinogen low	D-dimer usually normal* FVIII levels not affected
Microangiopathic haemolytic anaemia (e.g. HELLP, TTP)	Red cell fragmentation on blood film Microthrombi common Platelet count low	Bleeding uncommon Coagulation tests normal† ADAMTS13 below 10% in TTP
Hyperfibrinolysis	PT, APTT abnormal Fibrinogen low D-Dimer increased	Platelet count normal
Catastrophic antiphospholipid antibody syndrome	PT, APTT abnormal Platelet count low	Fibrinogen not low D-dimer normal‡
Massive transfusion	PT, APTT abnormal Fibrinogen low Platelet count low	D-dimer normal

* Unless additional disorders which increase the D-dimer coexist.
† Unless coexisting DIC.
‡ Some antibodies can affect the D-dimer results. HELLP, haemolysis, elevated liver enzymes, low platelet count (syndrome); TTP, thrombotic thrombocytopenic purpura.

between an increasing DIC score and mortality has been demonstrated. The optical light transmittance profile of the APTT, referred to as the biphasic waveform, correlates well with the overt DIC score and occurs independently of prolongation in the clotting times in patients with DIC. However, this is only available on some analysers.

Table 43.3 ISTH Sub-Committee of the Scientific and Standardisation Committee on DIC recommended scoring system for overt disseminated intravascular coagulation (DIC).

1 Risk assessment: does the patient have an underlying disorder known to be associated with overt DIC?
If yes, proceed
If no, do not use this algorithm
2 Order global coagulation tests (platelet count, PT, fibrinogen, soluble fibrin monomers or FDPs)
3 Score global coagulation test results
Platelet count ($\times 10^9$/L): >100, score 0; <100, score 1; <50, score 2
Elevated fibrin-related marker (e.g. soluble fibrin monomers or FDPs): no increase, score 0; moderate increase, score 2; strong increase, score 3
Prolonged PT (s): <3, score 0; >3 but <6, score 1; >6, score 2
Fibrinogen (g/L): <1 g/L score 1, fibrinogen ≥1 g/L score 0
4 Calculate score
5 Score ≥5 compatible with overt DIC. Repeat scoring daily

FDPs, fibrin degradation products; PT, prothrombin time.

Treatment

The mainstay of treatment of DIC is to remove the underlying cause. However, DIC often continues after appropriate treatment for the underlying condition. Supportive therapy with blood products may be necessary. Although the efficacy of blood product replacement has not been proven in randomised controlled trials, it is a biologically rational option to replace both thrombin-promoting and thrombin-opposing proteins, particularly when there is significant depletion of these factors in a patient who is either bleeding or at risk of bleeding. In those patients not bleeding, transfusion of platelets or plasma should not be undertaken based on laboratory results alone. There are no clinical or experimental data to suggest that platelet or plasma transfusions worsen the thrombotic process.

In patients with DIC and bleeding or at high risk of bleeding (e.g. after invasive procedures) and a platelet count less than 75×10^9/L, transfusion of platelets should be considered, especially because platelet function may be impaired. In bleeding patients with DIC and prolonged clotting times, fresh-frozen plasma (FFP) should be administered. If fluid overload is an issue, prothrombin complex concentrates (PCCs) could be considered, but these will only correct vitamin-K-dependent components. Activated PCCs may precipitate or worsen DIC. Severe hypofibrinogenaemia (<1.5 g/L) requires the use of cryoprecipitate or fibrinogen concentrate to correct.

The role of heparin in DIC remains controversial and unproven. A prophylactic dose of low-molecular-weight heparin is given to all patients with no evidence of active bleeding.

Since reduction of natural anticoagulants is often observed, trials using these agents have been conducted. However, no benefit has been found with activated protein C and its use is not recommended for DIC. Similar results were found with antithrombin infusions. The phase 3 SCARLET (Sepsis Coagulopathy Asahi Recombinant LE Thrombomodulin) trial was designed to determine the impact of soluble human recombinant thrombomodulin on 28-day mortality in patients with sepsis-associated coagulopathy. No difference was found in mortality between thrombomodulin and placebo while major bleeding was slightly more frequent in the thrombomodulin group.

Coagulopathy associated with COVID-19

Coronavirus disease (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The majority of patients who develop COVID-19 experience mild respiratory symptoms, but a significant minority require hospitalisation. A common complication of COVID-19 is thrombosis, primarily in the lungs but also in the different circulatory beds including both arterial and venous distributions. The epicentre of this infection is the lungs where an activated immune system can trigger thrombosis similar to other immunological disorders (termed 'immunothrombosis' or 'thromboinflammation'). Immunothrombosis can either be due to high viral load and the resulting vigorous immune response or to an exaggerated immune response. Thrombi usually commence in the pulmonary microvasculature which, if uncontrolled, can lead to pulmonary thrombi and spread of thrombi into the systemic circulation. Another reason for the high incidence of thrombi in COVID-19 is possibly the direct viral invasion of the endothelium (viral endothelialitis) which causes large amounts of ultra-large von Willebrand factor multimers to be released, although this remains contentious.

One of the most consistent laboratory findings in most hospitalised patients with COVID-19 is elevated D-dimers. Since D-dimers are a marker of clot breakdown, anticoagulation was applied to all inpatients with increasing doses considered for those with higher D-dimer values and critically ill. However, a proportion of D-dimers were from the intrapulmonary (extravascular) fibrinolysis and hence not from thrombi dissolution. In these cases, D-dimers correlated with mortality due to the worsening lung status from acute lung injury but not with thrombi formation. With respect to other laboratory parameters, severe thrombocytopenia is extremely rare and should raise the possibility of immune or drug-induced thrombocytopenia. PT and APTT are rarely abnormal except in late stages (Figure 43.2). Fibrinogen was markedly elevated in many patients but may be reduced in very severe cases. Several other tests have been suggested to correlate with COVID-19 severity including VWF/ADAMTS-13 ratio, complement activation markers, neutrophil extracellular traps and histones

Prevention of thrombosis is a crucial part of COVID-19 management. All hospitalised patients should receive prophylactic anticoagulation in the absence of any contraindications. Low-molecular-weight heparin is commonly chosen due to the ease of administration and good bioavailability. With respect to dosing, at the time of writing,

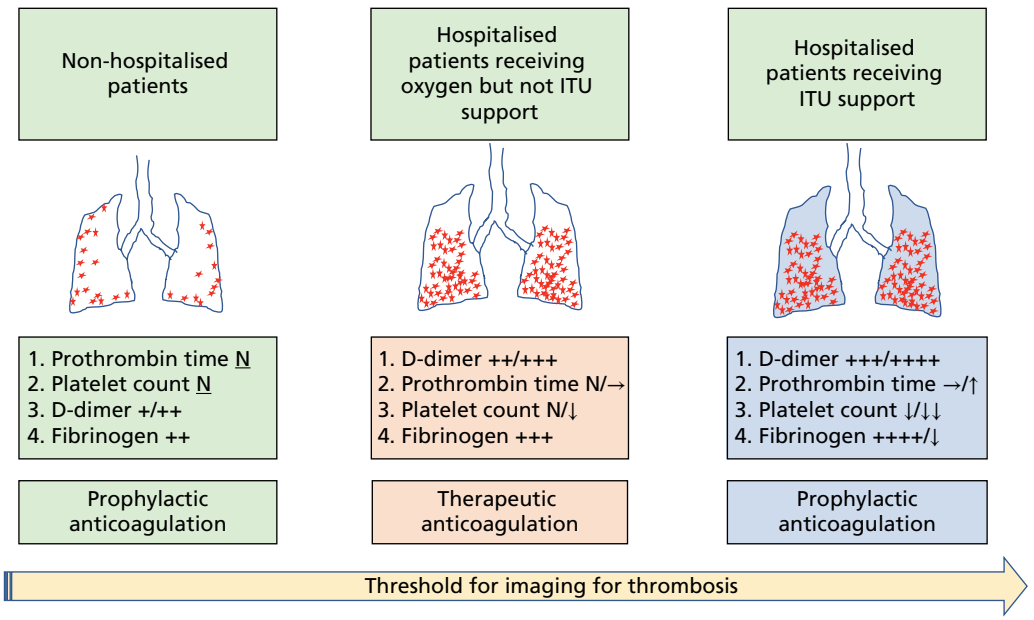

Figure 43.2 Changes in routine coagulation tests associated with COVID-19 infection.

randomised trials have shown therapeutic anticoagulation to increase mortality rates in patients requiring organ support or intensive care admission, while those who need supplemental oxygen but not yet receiving high-flow oxygen or ventilation benefit from therapeutic anticoagulation since it can reduce the need for organ support and mortality. A low threshold should be used for imaging to confirm thrombosis and therapeutic anticoagulation administered if proven to have thrombosis (Figure 43.2).

Changes in coagulation parameters vary with the severity of COVID-19 infection. The advice regarding anticoagulation is based on data available at the time of writing but this is a rapidly evolving field and management advice may change.

Haemostatic dysfunction in acute promyelocytic leukaemia

Acute promyelocytic leukaemia (APL), in particular the microgranular variant (AML-M3v), is associated with major coagulation disturbance, including DIC in at least 80% of cases. Although the introduction of all-*trans* retinoic acid (ATRA) and arsenic trioxide as differentiation agents has markedly reduced the rate of early haemorrhagic death and almost 90% of patients are cured, the 10% mortality from bleeding complications has not improved. Bleeding does not correlate with the clotting parameters, but with a high white cell count (see also Chapter 32).

The causes of coagulopathy and bleeding include hyperfibrinolysis (which is often the dominant process), DIC, thrombocytopenia, enhanced proteolysis and disruption of endothelial barrier integrity. APL blast cells express TF and annexin II which can act as a cell surface receptor for plasminogen and tissue plasminogen activator (tPA). Release of extracellular vesicles from blasts may also play a role. Cancer procoagulant, a cysteine proteinase detected on APL blast cells, can activate FX independent of FVII and is progressively reduced once patients have been commenced on ATRA. ATRA has also been demonstrated to inhibit vascular endothelial growth factor (VEGF) production, which indirectly limits TF production. Elastase, cathepsin-G and proteinase-3 are present in the granules of the APL blasts and can directly degrade fibrinogen, α_1 -antitrypsin, C1 esterase inhibitor and VWF, inducing the loss of high-molecular-weight multimers.

Management of the haemostatic abnormalities revolves around supportive care, including platelet transfusions to maintain a count of at least 30×10^9/L (or 50×10^9/L if the patient is bleeding) and the adequate replacement of fibrinogen to at least 1.5 g/L. The use of antifibrinolytic agents is supported by some evidence but remains controversial. Although markers of coagulation activation and fibrinolysis fall rapidly and completely following the start of ATRA therapy, there appears to be a slower resolution of procoagulant markers (up to 30 days). This may partly explain the clinical observation of thromboembolic events occurring in patients on ATRA, and prophylactic use of low-molecular-weight heparin or other anticoagulants should be considered once bleeding manifestations have settled.

Vitamin K and related disorders

Vitamin K metabolism

Vitamin K ('K' denoting *Koagulation*) is a group of lipophilic and hydrophobic vitamins that are needed for the post-translational modification of proteins. Vitamin K is a cofactor for gamma-glutamyl carboxylase, an enzyme that catalyses the carboxylation of glutamic acid (Glu) residues in several proteins (Figure 43.3). This reaction results in vitamin K 2,3-epoxide, which is recycled to reduced vitamin K by vitamin K epoxide reductase (VKOR), the enzyme inhibited by coumarin anticoagulants (Figure 43.3).

The Gla domains of the vitamin-K-dependent coagulant and anticoagulant proteins (FII, FVII, FIX, FX and proteins C and S) allow calcium-dependent binding to negatively charged phospholipids of activated cell membranes. This enhances the rate of the enzymatic reactions and localises them to the site of vessel injury. In the bones, carboxylation of the Gla protein osteocalcin is essential for incorporation of calcium into hydroxyapatite crystals. Retrospective studies suggest that long-term therapy with coumarin-based anticoagulants can affect vertebral bone density and fracture risk. Warfarin therapy is also associated with arterial calcification due to inhibition of matrix gamma-carboxyglutamate Gla protein.

Vitamin K deficiency

Vitamin K deficiency can occur at any age but is more common in infants because vitamin K does not cross the placenta, breast milk has low levels, there is low colonic bacterial synthesis and maternal ingestion of drugs that inhibit vitamin K. The clinical presentation of vitamin K deficiency in infants is bleeding. It was previously called 'haemorrhagic disease of the newborn' but is now termed vitamin K deficiency bleeding (VKDB). Supplementation of vitamin K is necessary to

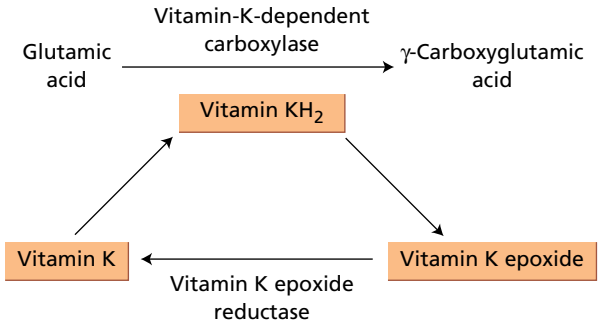

Figure 43.3 Vitamin K metabolism.

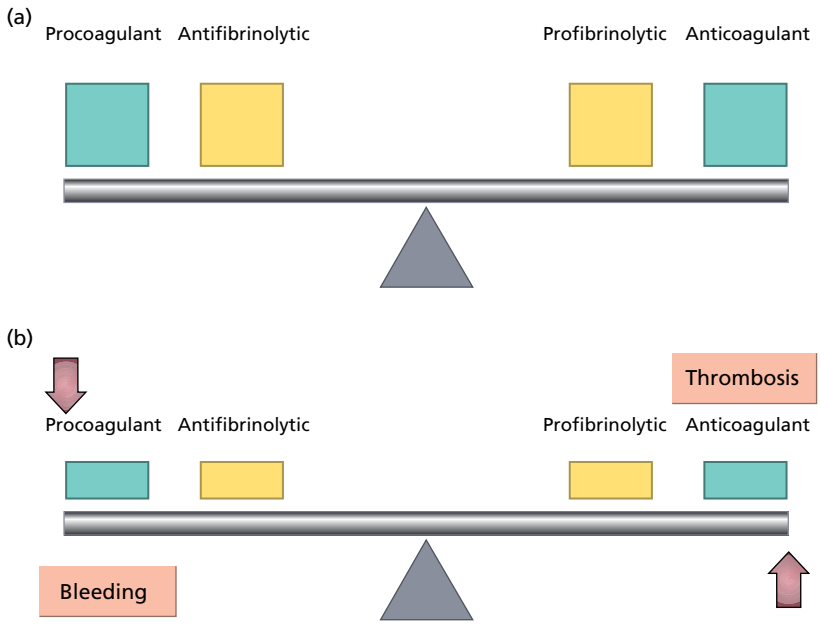

Figure 43.4 Haemostatic disturbance in liver disease. The haemostatic system in liver disease (b) compared with normal haemostasis (a). In healthy adults, the procoagulant and anticoagulant systems and the profibrinolytic and antifibrinolytic systems form a balance that is not easily disturbed. In patients with liver impairment, all four components are variably reduced and additional stimuli like infections or renal failure can upset the balance very easily, leading to either bleeding or thrombosis.

reduce the risk of bleeding, especially in exclusively breast-fed babies. A single intramuscular injection of vitamin K 1 mg prevents VKDB; however, if oral replacement is used, prolonged administration is required, although the exact dose is controversial.

In adults, vitamin K deficiency is uncommon due to recycling of the vitamin and an adequate gut flora. However, a poor dietary intake in combination with antibiotic therapy can cause deficiency. Other causes of vitamin K deficiency include malabsorption and cholestatic liver disease.

Vitamin-K-dependent coagulation factors in healthy full-term infants are about half of normal adult values. Adult values are reached by about 6 months, except for protein C, which does not reach adult levels until adolescence. Coagulation tests should be compared with age-matched reference ranges to distinguish physiological and pathological deficiencies.

VKDB in young children has been classified into early, classical and late types. The clinical features are shown in Table 43.4. The diagnostic criteria for VKDB include a PT more than 4 times control in the presence of at least one of: (i) a normal platelet count, normal fibrinogen level and absent fibrinogen degradation products, (ii) normalisation of coagulation tests after parenteral vitamin K administration or (iii) the presence of proteins induced by vitamin K absence or antagonism (PIVKA) in plasma. The presence of PIVKA without a coagulation deficit is a marker of subclinical vitamin K deficiency.

The treatment of a non-life-threatening bleed is with vitamin K_1 (phytomenadione) given slowly intravenously. A dose

Table 43.4 Features of vitamin K deficiency bleeding (VKDB).

	Early VKDB	Classical VKDB	Late VKDB
Presentation	First 24 hours of life	Days 2–7 of life	Day 8–6 months of life
Cause	Infants of mothers on vitamin-K-inhibiting drugs	Inadequate feeding	Cholestasis or malabsorption syndromes
Clinical presentation	Severe, with intracranial and intrathoracic bleeds	Milder, with gastrointestinal or umbilical bleeds being common	Severe, with high incidence of intracranial bleeds with 'warning bleeds' in up to one-third
Incidence of intracranial haemorrhage	High	Rare	Very high

of 1–2 mg is enough to fully correct the deficiency in infants aged up to 6 months, higher doses offering no advantage in efficacy or speed of reversal. There have been reports of anaphylactic reactions although these are rare. The PT should improve within 48 hours and if this has not occurred after three doses, continuation of vitamin K is unlikely to help in most cases and an alternative cause of the prolonged PT sought. Oral replacement may be used if there is no active bleeding and absorption is normal. Patients with severe bleeding should be treated with FFP or PCC.

Poisoning following the accidental ingestion of large doses of warfarin or compounds with similar properties and long half-lives is suggested by poor correction of PT or International Normalised Ratio (INR) with normal doses of vitamin K. Serum assays of warfarin concentration can be undertaken for confirmation.

Haemostatic disturbance in liver disease

Almost all procoagulant factors, natural anticoagulants and inhibitors of coagulation are synthesised in the liver (Figure 43.4). The liver is also involved in the clearance of activated clotting factors. The effect of liver disease on haemostasis is therefore complex and a balance between procoagulant and antithrombotic changes (Figure 43.4; Table 43.5). Routine coagulation tests such as PT and APTT are commonly prolonged in liver disease but are only sensitive to decreases in procoagulant factors and hence are liable to over-estimation of the haemostatic defect. Patients with liver disease have similar thrombin generation to healthy control individuals when tests are performed in the presence of thrombomodulin to make them sensitive to protein C levels.

Acute hepatitis

Patients with acute hepatitis are often thrombocytopenic (platelets $100–150 \times 10^9$/L). The mechanism for this may be immune, due to concurrent hypersplenism or to DIC. Platelet function may be impaired in acute hepatitis, although this is unlikely to be of clinical significance. In acute liver disease, biosynthesis of clotting factors is impaired, and this may be reflected in a prolongation of coagulation tests. The PT and FV, FVII, antithrombin and protein C levels are the most sensitive to hepatic biosynthetic dysfunction. The PT and FVII level predict survival in acute liver disease. The plasma fibrinogen may be raised in acute hepatic disturbance as part of an acute-phase response and a low fibrinogen is associated with a poor prognosis. A pattern of reduced fibrinogen and increased fibrin degradation products seen in acute liver disease may be due to hyperfibrinolysis.

Chronic liver disease

Thrombocytopenia

Thrombocytopenia and platelet dysfunction are common in chronic liver disease. Mild to moderate thrombocytopenia is seen in about 30% of patients with chronic liver impairment, 76% in cirrhosis and about 90% of patients with terminal liver disease. In patients with cirrhosis, this is frequently related to hypersplenism, secondary to portal hypertension. In addition, folate deficiency and the direct toxic effect of ethanol on megakaryocyte function may contribute.

A more profound thrombocytopenia in patients with hepatitis C virus (HCV) infection may be autoimmune and, although the response to steroids may be poor, eradication of HCV may be associated with an increased platelet count. Thrombopoietin receptor agonists increase platelet counts to more than 100×10^9/L in the majority of patients with cirrhosis associated with HCV infection.

A variety of platelet aggregation abnormalities has also been demonstrated in liver disease although their clinical significance is debated. VWF may be increased 10-fold and can

Table 43.5 Causes of increased bleeding tendency or coagulation abnormalities in liver disease.

Decreased coagulation factors
Decreased synthesis
Vitamin K deficiency
Increased clearance
Abnormal coagulation factors due to hypocarboxylation
Decreased platelets
Sequestration in spleen or liver
Thrombopoietin deficiency
Destruction due to toxins or antiplatelet antibodies or toxins
Toxic effect of alcohol on marrow
Dysfunctional platelets
Altered platelet arachidonic acid metabolism
Defective signal transduction
Storage pool deficiency
Abnormalities of the platelet glycoproteins
Increased platelet inhibitors nitric oxide and prostacyclin
Hypofibrinogenaemia
Impaired synthesis
Loss into extravascular spaces (ascites)
Increased catabolism
Loss due to massive haemorrhage
Abnormal fibrinolysis
Increased release and decreased clearance of tissue plasminogen activator
Decreased synthesis of α_2 -antiplasmin, plasminogen and thrombin-activated fibrinolysis inhibitor
Infections
Endogenous heparinoids: anti-FXa activity
Renal failure
Sinusoidal endothelial cell dysfunction
Hypothermia

support platelet adhesion despite reduced aggregation. However, desmopressin did not show any efficacy in reducing blood loss in patients undergoing partial liver resection or liver transplantation.

Procoagulant and anticoagulant factors

Coagulation factor and natural anticoagulant synthesis are affected by chronic liver disease. FIX is usually reduced less than FII, FVII, FX and protein C. The levels of these factors, especially FVII, falls proportionately with increasing severity of disease, and FVII is an independent predictor of survival (level below 34% associated with 93% mortality). Chronic liver impairment leads to vitamin K deficiency, mainly by decreased absorption from the gut as a result of reduced bile salt secretion in cholestasis (parenteral vitamin K responsive) and decreased synthesis in parenchymal disease (parenteral vitamin K non-responsive). The PT has been incorporated into prognostic indices of chronic liver disease, such as the Child–Pugh and Mayo End-Stage Liver Disease scores. Despite the prolongation of clotting tests such as PT and APTT, bleeding after liver biopsy or other invasive procedures in patients with cirrhosis is rare, suggesting that these tests are not on their own indicative of the haemorrhagic tendency. The levels of anticoagulant proteins, antithrombin and proteins C and S are reduced in a similar manner to procoagulant factors, potentially retaining the net balance in haemostasis. It is common practice to use the INR instead of the PT to access patients with liver disease. However, the INR is Standardised for patients taking vitamin K antagonists and its use in patients who have liver disease would require determination of an international sensitivity index (ISI) relevant to liver disease.

Bacterial infection and variceal bleeding may be associated with increased heparinoid and anti-Xa activity.

Fibrinogen

Most patients with stable chronic liver disease have normal or increased fibrinogen, although in advanced disease fibrinogen falls. This may be due to impaired synthesis, loss into extravascular spaces (ascites), increased catabolism or massive haemorrhage. Some patients develop dysfibrinogenaemia due to increased activity of sialyltransferase expressed by immature hepatocytes generated during hepatic injury, which leads to low-molecular-weight fibrinogen with abnormal α-chains and higher sialic acid content. Dysfibrinogenaemia is reflected by a prolonged TT and/or reptilase time.

Fibrinolysis

Cirrhosis may be associated with accelerated fibrinolysis due to decreased clearance of tPA, and decreased synthesis of α_2-antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI). Hyperfibrinolysis has also been found to be a predictor of the first episode of upper gastrointestinal bleeding in cirrhotic patients with portal hypertension. On the other hand, life-threatening haemorrhage in patients with liver disease is more often related to portal hypertension (the localised coagulation problem used to be termed 'accelerated intravascular coagulation and fibrinolysis') than to net dysfunction of the clotting cascade, wherein the varices play a major part. A rebuttal to the concept of hyperfibrinolysis in cirrhosis comes from the fact that markers for hyperfibrinolysis such plasmin-antiplasmin complexes may be spuriously elevated in patients as a consequence of defective clearance and do not equate to hyperfibrinolysis as defined in other clinical states like trauma. Renal failure is also common in advanced liver disease (hepatorenal syndrome), which can exacerbate a bleeding risk.

Management of haemostatic impairment

The management of a bleeding patient with hepatic dysfunction depends on the site of bleeding and the haemostatic dysfunction. Clotting function should be assessed by means of the PT, APTT, TT and fibrinogen and D-dimer levels, although the results should be interpreted in the clinical context before transfusions are undertaken. Vitamin K should be administered intravenously (10 mg daily for 3 days) to aid biosynthesis of vitamin-K-dependent factors. The widespread practice of managing the coagulopathy of liver disease with transfusions of FFP or platelets should only be considered in the presence of active bleeding. Modification of risk factors for bleeding other than laboratory values, such as sepsis, renal impairment and anaemia should be considered if possible.

There are no evidence-based guidelines to treat active bleeding or establish safe coagulation levels for liver biopsy and similar procedures although consensus guidelines recommend a platelet count of at least 50×10^9/L (transjugular route is safer than percutaneous for liver biopsy). Based on the lack of evidence that increasing platelet counts can decrease bleeding risk, the society of liver disease recommends performing both low- and high-risk procedures without correcting the platelet count. Thrombopoietin receptor agonists have been trialled in this setting to increase the platelet count and may reduce the need for platelet transfusion.

Correction of the coagulopathy is particularly important before the placement of an intracranial pressure transducer in patients with hepatic encephalopathy. Spontaneous intracranial bleeding has been reported in such patients, though rarely, and this remains one of the principal concerns regarding abnormal coagulation in liver disease. In patients with evidence of increased fibrinolysis, antifibrinolytic drugs such as tranexamic acid should be considered.

Liver transplantation

The changes in the balance of haemostasis seen during liver transplantation are complex and multifactorial and can be due to surgical and non-surgical causes. Traditionally, the process of liver transplantation is split into three stages: stage I, the pre-anhepatic stage, which ends with the occlusion of the recipient's hepatic blood flow; stage II, the anhepatic phase, which ends with the re-perfusion of the donor liver; and stage III, the reperfusion and neohepatic period.

The risk of bleeding in the pre-anhepatic stage is related directly to the preoperative haemorrhagic risk related to the underlying liver disease. A reduction in procoagulant factors may be seen, especially if large blood losses necessitate transfusion, leading to dilution. During the anhepatic stage, studies have reported enhanced fibrinolytic activity. Reperfusion of the liver during the postanhepatic phase is associated with a dramatic increase in fibrinolysis in almost three-quarters of patients. Usually, hyperfibrinolysis subsides within an hour, but in a damaged donor liver, sustained increased fibrinolytic activity may be observed. The endothelium of the donor liver is an important source of tPA; the ischaemic damage to the graft during preservation may explain the dramatic increase in plasminogen activators. After re-perfusion, the release of heparin-like compounds has also been observed. In the postoperative period, a reduction in platelet count is related to platelet activation and blood loss, in addition to low thrombopoietin levels. The levels of this hormone increase significantly on the first day after liver synthetic function is restored, with the platelet count normalising in 2 weeks.

There is little evidence that one can predict the transfusion requirement of a patient by examining the preoperative coagulation results. The profound coagulation abnormalities seen in fulminant hepatic failure should be corrected preoperatively; however, correction of a mild coagulopathy in a patient with chronic liver disease is less likely to be of benefit. During surgery, the aim is to transfuse blood components to prevent the development of intractable coagulopathy. Near-patient tests of haemostasis performed on whole blood are often used in theatre to complement the tests performed in the coagulation laboratory. Many departments use algorithms based on coagulation and near-patient testing to guide FFP, red cell, cryoprecipitate and platelet transfusion during the procedure. Aprotinin can be administered to counteract the increase in fibrinolysis observed.

Hypercoagulability in liver disease

Tipping off the balance in the complex interplay between endogenous procoagulants and the anticoagulant system in liver disease can lead to a hypercoagulable state, in contrast to the common assumption of an 'auto-anticoagulated state'. The hypercoagulable events may be clinically evident, as with portal vein thrombosis and venous thromboembolism, but in the microvasculature can contribute to porto-pulmonary hypertension, liver fibrosis, thrombosis of extracorporeal circuits and progression of non-alcoholic steatohepatitis to cirrhosis. Hypercoagulation in liver disease may be related to poor flow, endothelial dysfunction and vasculopathy associated with a chronic inflammatory state, increased levels of FVIII and VWF, or decreased synthesis of the naturally occurring anticoagulant proteins. Hepatic fibrogenesis may be caused by tissue ischaemia and direct thrombin-mediated stellate cell activation by PAR-1 cleavage.

Haemostatic disturbance in renal disease

Bleeding is a frequent cause of morbidity and mortality in patients with renal failure. Common manifestations are gastrointestinal bleeding from angiodysplasia and peptic ulcers, as well as prolonged bleeding from skin puncture sites. Subdural haematomas and haemorrhagic pericarditis are also seen, although bleeding at the time of renal biopsy is rare. Bleeding is seen despite normal or elevated circulating levels of coagulation factors, suggesting that platelet abnormalities are the likely cause. This is supported by the finding of a prolonged bleeding time and reduced platelet aggregation to various agonists. Severe thrombocytopenia (platelets $<50 \times 10^9$/L) secondary to renal failure is rare and its presence should suggest concomitant conditions such as HCV infection or vasculitis.

Platelet dysfunction with renal failure is multifactorial and can be divided into: (i) intrinsic platelet defects, (ii) abnormal interaction of platelets with the endothelium, (iii) effects of uraemic toxins, (iv) effects of anaemia on platelets and (v) dialysis-related. Intrinsic platelet abnormalities reported in association with uraemia are shown in Table 43.6. The interaction between uraemic platelets and the endothelium can be markedly reduced, due partly to impaired VWF binding to platelets and increased endothelial production of nitric oxide, an inhibitor of platelet aggregation. Uraemic patients with prolonged bleeding times also have raised prostaglandin (PG)I_2 levels.

Anaemia-related haemostatic dysfunction is primarily a result of reduced displacement of platelets to the vessel wall by red cells.

Table 43.6 Platelet abnormalities reported in uraemia.

Decreased GPIb complexes
Reduced serotonin and ADP in the delta granules
Increased levels of cyclic AMP
Defective ristocetin-induced platelet aggregation
Abnormal mobilisation of free cytoplasmic calcium in response to agonists
Reduced release of arachidonic acid from membrane phospholipids
Decreased conversion of arachidonic acid to thromboxane A_2
Abnormal dense-granule and α-granule secretion
Abnormal cytoskeletal assembly
Deficient tyrosine phosphorylation

Dialysis can improve bleeding symptoms. Antiplatelet drugs, antibiotics, oral anticoagulants (especially those that can accumulate in renal failure) and heparin may contribute to the bleeding risk.

The bleeding time better correlates with clinical bleeding than tests of renal function, although measurement is rarely clinically useful. Adequate dialysis will improve symptoms and the bleeding time in most patients. Intravenous, subcutaneous or intranasal administration of desmopressin in conjunction with antifibrinolytics has also been used successfully to control uraemic bleeding. Although the response is brief (4–8 hours), the rapid onset of action is beneficial. Care is required to avoid hyponatraemia. Desmopressin is thought to improve platelet function by the enhanced release of endothelial VWF multimers and also by increasing phospholipid expression.

Correction of anaemia with erythropoietin so that the haematocrit exceeds 30% improves platelet interaction with the vessel wall and has also been shown to increase the number of reticulated platelets. Other potentially beneficial effects of erythropoietin include improved platelet intracellular calcium mobilisation, increased expression of GPIb and improved platelet signal transduction.

Pregnancy-related haemostatic dysfunction

Normal pregnancy is associated with physiological changes in haemostasis, with increased levels of procoagulant proteins such as fibrinogen, VWF and FVIII and a fall in anticoagulants such as protein S. These changes partly contribute to the increased risk of venous thromboembolism during pregnancy.

Obstetric haemorrhage is the most common cause of severe pregnancy-related morbidity. The cause of bleeding is often multifactorial, with a combination of physical and acquired haemostatic defects. Consumption leads to depletion of platelets and coagulation factors, particularly fibrinogen. An aggressive consumptive coagulopathy is seen with amniotic fluid embolus which is often fatal. Consumptive coagulopathy may also be triggered by placental abruption, intrauterine fetal death, pre-eclampsia and acute fatty liver of pregnancy. Once bleeding and resuscitation has started, a dilutional coagulopathy may exacerbate the haemostatic failure and contribute to the ongoing bleeding.

Treatment requires rapid recognition of bleeding and a coordinated response from obstetricians, midwives, anaesthetists and haematologists. Obstetric management should be optimised. Assessment of haemostatic failure requires an urgent FBC and a coagulation screen that includes a Clauss fibrinogen because the PT and APTT may be normal despite a low fibrinogen. Thromboelastometry in combination with a validated algorithm is becoming more commonly used because laboratory coagulation tests are often too slow to manage patients adequately. In the absence of thromboelastometry empirical blood product replacement may be required. In patients with severe haemostatic failure, a standard dose of FFP (15 mL/kg) is unlikely to be adequate to correct the haemostatic defect and the use of larger volumes should be anticipated. Platelets should be maintained above 75×10^9/L and cryoprecipitate used to maintain fibrinogen above 2 g/L if bleeding is ongoing. Some centres use fibrinogen concentrate in obstetric haemorrhage to rapidly correct hypofibrinogenaemia. The use of rFVIIa or prothrombin complex concentrate is not recommended in guidelines.

As soon as bleeding has been controlled, the patient is likely to be at high risk of venous thromboembolism; this should be assessed and appropriate thromboprophylaxis started.

Haemostatic dysfunction associated with cardiopulmonary bypass surgery

Coronary artery bypass graft (CABG) surgery is associated with excessive bleeding in about 10% of cases. Re-operation for bleeding is required in 3–10% of cases. Bleeding is more common with revision procedures and prolonged time on bypass. A surgically correctable source of bleeding may be found. Bleeding due to haemostatic disturbances can be patient-related or CPB related. Antiplatelet, anticoagulant or fibrinolytic drugs, particularly at the time of emergency CABG after stenting procedures, commonly contribute to perioperative bleeding. Despite its antiplatelet activity, aspirin has not been demonstrated to cause increased blood loss after CABG.

Haemostatic abnormalities associated with CPB are related to the interaction of blood with the extensive non-endothelial bypass surfaces and retransfusion of pericardial blood leading to contact activation. All coagulation factors except factor VIII are reduced during CPB. CPB decreases both the number and function of platelets. Platelet counts fall by 25–60% within 15 min of first passage of blood through the primed CPB circuit, in association with a prolonged bleeding time. The platelet count rarely falls below 100×10^9/L and persistent or profound thrombocytopenia should prompt consideration of alternative causes, including heparin-induced thrombocytopenia.

The observed changes in platelet function in patients undergoing CPB are due to modification of membrane components with loss of receptors, decrease in granule contents, aggregation to fibrinogen adsorbed onto the bypass circuit, mechanical trauma, exposure to hypothermia and heparin. Following CPB, platelet function returns to normal within 1 hour, although the platelet count may take several days to normalise. Coagulation factors fall because of haemodilution, which can be exacerbated by use of cell-salvage systems. Fibrinolysis is also enhanced by returned blood from the pericardiotomy suction.

Blood component administration in patients with excessive bleeding related to CABG is often empirical, with replacement of FPP and platelets. Near-patient whole-blood thromboelastometry has been used to assess haemostasis and fibrinolysis and shown to help rationalise blood product usage. Heparinase cups are often used to assess haemostasis in heparinised samples. The off-licence use of PCCs and fibrinogen concentrates has also been reported. Randomised studies do not support the use of

rFVIIa and it has been associated with increased thrombotic adverse events. Tranexamic acid and aprotinin are often used to inhibit fibrinolysis.

Haemostatic dysfunction associated with trauma

Acute traumatic coagulopathy (ATC) has been described to occur early during severe trauma and is caused by the trauma directly, shock and endothelial activation. It is characterised by increased activated protein C secondary to soluble thrombomodulin, inhibition of FV, dysfibrinogenaemia and hyperfibrinolysis. As bleeding and resuscitation progress a dilution coagulopathy contributes to the overall trauma-induced coagulopathy (TIC) (Figure 43.5). The aetiology of TIC is multifactorial and the important initiators include tissue injury with tissue factor exposure and increased thrombin generation, early reduction of fibrinogen, physiological and therapeutic haemodilution of coagulation factors and platelets, inflammation, hypothermia and acidosis. Recent work exploring mechanisms suggest tissue injury and trauma-related shock activating the various aspects of the haemostatic system, especially the vascular endothelium. In addition, the host response includes activation of the immune system leading to immunothrombosis. A varying balance between fibrinolysis and antifibrinolytic factors also comes into play with a predominant hyperfibrinolysis state due to increased tPA and reduced PAI-1 (usually early) leading to bleeding while a balance towards the antifibrinolytic pathway (usually later and after the initial hyperfibrinolysis) predisposes to thrombosis. Tranexamic acid is therefore recommended to be given before 3 hours; after this time, its use has been associated with worsening outcomes. Viscoelastic testing may be helpful in delineating this differential activation and may guide appropriate therapy including that of blood products.

Massive blood loss and resuscitation contributes to deficiencies of haemostatic factors and up to half of the fibrinogen and one-third of platelets may be lost before treatment of the injury has begun. The presence of an abnormal coagulation test on arrival in the emergency department correlates with the severity of injury and the mortality rate.

Guidelines on triggers for blood product support are evolving and different specialties have produced variable advice; an example is given in Table 43.7. Many trauma centres use thromboelastometry to guide blood product replacement. Recent observations, predominantly based on experience of battlefield trauma, have prompted recommendations for aggressive and early plasma and platelet replacement with a ratio of 1:1:1 for red cells, FFP and platelets. Issues such as hypothermia and acidosis need to be addressed by using prewarmed fluids and extracorporeal warming devices and appropriate resuscitation.

Trauma and shock cause endothelial dysfunction and contribute to acute traumatic coagulopathy. Resuscitation may result in haemodilution and contribute to trauma-induced coagulopathy. Hyperfibrinolysis is common early in severe trauma but antifibrinolytic mechanisms take over later during the process.

Coagulopathy in massive blood loss

Massive blood loss is usually defined as one blood volume (about 5 L in an adult) in 24 hours or 50% blood volume in 3 hours or more than 150 mL/min. The associated coagulopathy is complex and multifactorial. It is partly dilutional, as blood is replaced with volume expanders and red cells, but DIC, hypoxia, hypothermia and acidosis contribute in some clinical situations. Haemostatic management should be to maintain a platelet count of at least 75×10^9/L, a threshold that is likely to be reached after a two-blood-volume

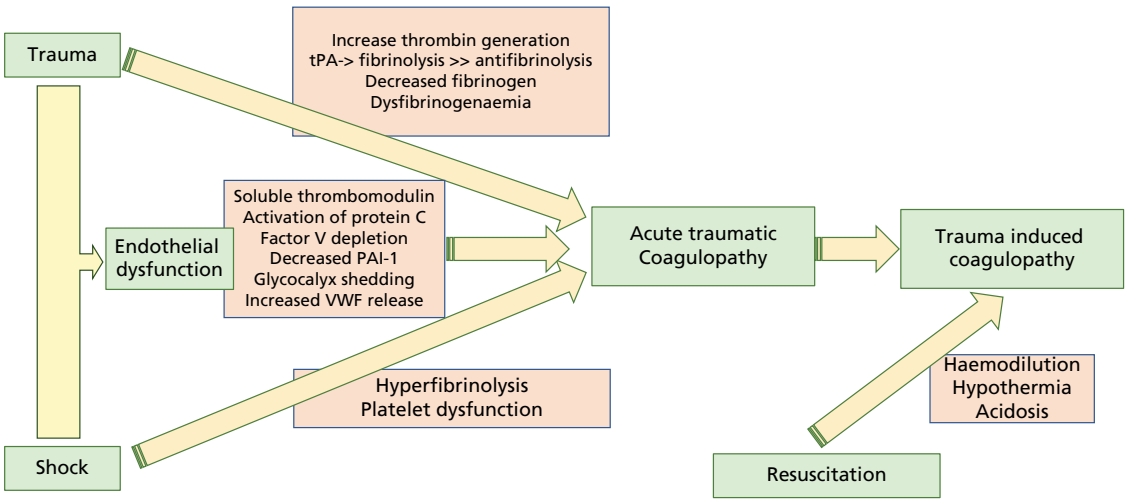

Figure 43.5 Mechanisms of haemostatic dysfunction during severe trauma.

Table 43.7 Guide to blood product replacement in massive blood loss.

1 Give tranexamic acid before 3 hours
2 Control bleeding using surgical and/or radiological interventions
3 Restore an appropriate circulating blood volume
4 Control exacerbating factors: hypothermia and acidosis
5 Blood product support as detailed below
Red cells
Use O-negative red cells first
If no record of red cell antibodies, ABO- and Rh-compatible cross-matched blood should be available within 30 min (maximum 45 min)
Replace red cells as required to maintain circulating blood volume
Use blood warmer to avoid hypothermia
Fresh-frozen plasma
Transfuse FFP in fixed ratio with red cells to prevent coagulopathy to maintain PT and APTT <1.5 times normal
If PT and APTT >1.5 times normal FFP at doses >15 mL/kg will be required
Platelet transfusion
Transfuse platelets in fixed ratio with red cells to maintain platelet count $>50 \times 10^9$/L
Fibrinogen
Maintain at least >1.5 g/L
Cryoprecipitate (dose: two donation pools)
Fibrinogen concentrate (50 mg/kg will increase fibrinogen by about 1 g/L)

haemorrhage. A higher platelet count of about 100×10^9/L will be needed in major trauma or central nervous system bleeding. Fibrinogen should be maintained above at least 1.5 g/L, a level that is likely to be reached after about a 1 to 1.5-blood-volume haemorrhage, assuming no significant additional consumptive coagulopathy. FFP should be transfused to maintain PT and aPTT less than 1.5 times normal.

Haemostatic therapy is to infuse platelets and FFP in volumes likely to maintain a safety margin above these critical levels (see also Chapter 19). Hypofibrinogenaemia unresponsive to FFP may require cryoprecipitate or fibrinogen concentrate. It is important to predict ongoing dilution and consumption of coagulation factors and to replace these expectantly if bleeding continues. Regular measurement of FBC and coagulation screens (including Clauss fibrinogen) are important for monitoring replacement treatment, although often these are not available rapidly enough to guide initial management and empirical treatment may be required in the early phases (see Table 43.7). Many centres are using thromboelastometry-guided treatment in this situation.

Bruising

Purpura simplex (normal easy bruising)

Distinguishing normal from pathological bruises may be difficult and individuals have variable thresholds for presenting for medical review. If bruising is not associated with other symptoms suggestive of a bleeding disorder and routine tests of coagulation (including VWF levels and platelet number) are normal, the patient can be reassured. Drugs such as non-steroidal anti-inflammatory agents or selective serotonin reuptake inhibitors are sometimes implicated.

Non-accidental bruising

Bruising is a common feature of non-accidental injury in both children and adults. Bruises that affect unusual sites, are in different stages of maturation, or shaped like a hand or instrument should raise concern. Any bruising in a non-mobile baby should be investigated although this may be seen in severe haemophilia. In the case of children, appropriate liaison with child safeguarding agencies should be undertaken, although the possibility of an underlying congenital or acquired bleeding disorder should also be thoroughly investigated. Self-harm may also present with bruising and should be suspected if the pattern of bruising is atypical. Deliberate ingestion of anticoagulants and long-acting vitamin K antagonists is also possible.

Senile purpura (atrophic or actinic purpura) and steroid-related purpura

Bruising is more common in elderly people due to atrophy of subcutaneous tissues and loss of collagen and elastin fibres in subcutaneous tissues. Blood vessels in the skin can be broken by minor trauma or shearing forces and typically purpura are seen on hands and forearms. If routine tests of platelet number and coagulation are normal, no further investigation is required. Long-term steroid use is also associated with bruising secondary to atrophy of collagen fibres supporting blood vessels.

Painful bruising syndrome (psychogenic purpura)

Diamond–Gardner syndrome is a rare condition most commonly seen in women with psychological problems. It is also called painful bruising syndrome and psychogenic purpura. It presents with painful ecchymotic lesions, mostly on the extremities and the face and rarely on less accessible parts of the body. The skin lesions can be preceded by paraesthesia or pain and are usually reported after surgery or minor trauma sometimes at site distant from the surgery. The usual tests for haemostasis reveal no abnormalities. The cause of the bruising is poorly understood, although Diamond and Gardner suggested the

possibility of a local reaction to the patient's own red cell stroma and gave it the name 'autoerythrocyte sensitisation syndrome'.

Scurvy

Scurvy is caused by a lack of vitamin C (ascorbic acid), which is required for the hydroxylation of prolyl and lysyl residues in the formation of mature collagen. Vitamin C deficiency renders the collagen unable to self-assemble into rigid triple helices, resulting in blood vessel fragility and poor wound healing. The characteristic clinical finding is perifollicular haemorrhage but large ecchymoses can occur on the legs and trunk. Follicular hyperkeratosis and corkscrew hairs are other cutaneous features. Bleeding gums are common. Scurvy is more common in people with poor nutrition, especially if dependent on alcohol. The diagnosis is clinical, although plasma or leucocyte vitamin C can be measured. A prompt response to vitamin C treatment is also diagnostic.

Inherited disorders of collagen and elastic fibres

Ehlers–Danlos syndrome (EDS) is a group of dominantly inherited disorders of connective tissue characterised by lax hyperelastic skin, joint laxity, spontaneous dislocation and poor wound healing. There are six subtypes and subtype IV is associated with severe varicosities and spontaneous rupture of major blood vessels. Bruising is seen in 90% of patients and has also been reported in people with lax joints who do not fulfil the criteria of EDS. Although mild platelet and coagulation factor dysfunction has been reported in association with EDS, they are likely to be coincidental. Some patients have a prolonged bleeding time and this can respond to desmopressin. Other disorders such as osteogenesis imperfecta may also be associated with bruising. Cutis laxa is not associated with abnormal bruising.

Haemostatic dysfunction associated with vasculitis

The vasculitides are a heterogeneous group of disorders characterised by inflammation within blood vessel walls of different organs. It is classified by the size of the blood vessel involved and can be associated with both thrombosis, for example, Behçet's disease (see Chapter 57), and haemorrhage. The classic presentation of Henoch–Schönlein purpura is palpable purpura without thrombocytopenia or coagulopathy, arthritis/arthralgia, abdominal pain and renal disease. Henoch–Schönlein purpura is more commonly associated with bleeding than thrombosis.

Arteriovenous malformations

Hereditary haemorrhagic telangiectasia

Hereditary haemorrhagic telangiectasia (HHT) is a dominantly inherited, highly penetrant, familial disease in which telangiectasia develop on the skin and mucous membranes. Large arteriovenous malformations may occur in solid organs. International consensus diagnostic criteria have been developed based on epistaxis, mucocutaneous telangiectasia, visceral lesions and an affected first-degree relative.

Mutations in the *ENG* gene (encoding endoglin) and *ACVLR1* gene (encoding activin receptor-like kinase 1) have been described. These genes encode proteins that are involved in signalling by the transforming growth factor (TGF)-β superfamily. Increased levels of VEGF have been demonstrated in HHT. Genetic testing may be useful to inform asymptomatic family members or in people in whom the diagnosis is unclear.

Cerebral, pulmonary and neurological involvement is described more commonly with *ENG* mutations, whereas liver involvement has been associated with *ALK1* mutations, although members of the same family can have variable phenotypes. Typical telangiectatic lesions are red 0.5–3 mm spots that blanche with pressure and are often noted on the skin of face and lips and mucosal surfaces of the tongue and mouth. Lesions may not be present in young children and progress with increasing age. Symptoms range from cosmetic, recurrent epistaxis and mouth bleeding to iron deficiency and intracranial haemorrhage. Septic emboli may occur and antibiotic prophylaxis at the time of invasive procedures such as dental extraction is recommended. Occasionally bleeding can occur from internal organs without visible lesions.

Virtually all internal organs can be affected. The lungs develop pulmonary arteriovenous fistulae in about 30% of patients, leading to haemoptysis, sometimes catastrophic, hypoxia, dyspnoea, pulmonary hypertension, high-output cardiac failure and clubbing. Transient ischaemic attacks, cerebral infarction and systemic emboli due to right-to-left shunting occur in about half of the patients with pulmonary arteriovenous fistulae. Pulmonary arteriovenous fistulae may worsen during pregnancy. Liver involvement is present in the majority of patients and, though often asymptomatic, can lead to cirrhosis, portal hypertension and high-output cardiac failure. Conjunctival telangiectasia may cause benign bleeds, while more serious retinal bleeds can occur. Intracranial haemorrhage is associated with arteriovenous fistulae, but embolisation, including septic emboli, may also cause cerebral symptoms.

Options for symptomatic lesions include local treatment, such as laser therapy of skin and mucosal telangiectasia and embolisation of arteriovenous malformations in lung or nervous system. Systemic treatment with oestrogens and tranexamic acid may reduce bleeding symptoms. If successful, liver transplantation may lead to reversal of cardiopulmonary changes. Experimental treatments include thalidomide and bevacizumab to inhibit VEGF.

Kasabach–Merritt syndrome

Kasabach–Merritt syndrome is a rare condition in which a vascular tumour (Kaposiform haemangioendothelioma) is associated with thrombocytopenia, hypofibrinogenaemia and

bleeding, which can be life-threatening. The thrombocytopenia in Kasabach–Merritt syndrome is presumed to be due to platelet adhesion to the abnormally proliferating endothelium. This leads to activation of platelets with secondary consumption of clotting factors, including VWF, resulting in systemic haemostatic failure. Excessive flow and shear rates secondary to arteriovenous shunting also contribute and microangiopathic haemolysis may be seen. The platelet count is often less than 20×10^9/L and the platelet half-life dramatically reduced. Intralesional bleeding can cause rapid enlargement of the haemangioma and can worsen the consumptive coagulopathy. Intralesional thrombosis may rarely occur, causing spontaneous resolution of some lesions. Management involves supportive care and, if possible, removal of the lesion. Steroids, interferon, chemotherapy with vincristine, radiotherapy, embolisation and antiangiogenic agents have been tried.

Microthromboembolic disease

Cholesterol embolism

Cholesterol embolism is a complication of widespread atherosclerotic disease. Rupture of an atherosclerotic plaque can occur spontaneously or, more commonly, at the time of vascular surgery or invasive procedures. Anticoagulant or fibrinolytic therapy can weaken thrombi that usually prevent the release of cholesterol crystals. The characteristic presentation is with limb small vessel occlusion, sometimes called purple toe syndrome, with well-preserved peripheral pulses, which may occasionally lead to gangrene. Another common skin finding is livedo reticularis, where the cutaneous venous plexus becomes visible because of increased amounts of desaturated venous blood. Organ involvement from cholesterol emboli is dependent on the vascular supply, with renal ischaemia being the commonest. The diagnosis is often missed unless the occurrence of the clinical features is related to the triggering procedure. Fundoscopy can reveal the presence of retinal cholesterol crystals (Hollenhorst plaques) in about 25% of cases. Biopsy of the cutaneous lesion or the affected organ is required for definitive diagnosis. The prostacyclin analogue iloprost has been used successfully in painful cutaneous necrotic lesions and renal insufficiency in a single report.

Fat embolism syndrome

Fat embolisation is characterised by release of fat droplets into the systemic circulation after a traumatic or iatrogenic event. Its incidence depends on the bone involved (fractures of the femoral shaft >tibia or fibula >neck of femur), isolated or multiple fractures, age (10–40 years) and gender (occurs more in males). Although more common after lower limb fractures, fat embolism syndrome can also occur after liposuction, bone marrow harvesting, total parenteral nutrition, sickle cell crisis and pancreatitis. Classically, it presents with the triad of respiratory distress, mental status changes and petechial rash 24–48 hours after pelvic or long-bone fracture. The rash is pathognomonic and is seen usually on the conjunctiva, oral mucous membranes and upper body, possibly due to embolisation of fat droplets accumulating in the aortic arch. The diagnosis is made clinically because laboratory and radiographic diagnosis is non-specific and inconsistent. Thrombocytopenia is common, due to platelet activation and consumption into the thrombi. Management is supportive to ensure haemodynamic stability. Aspirin and corticosteroids have also been shown to be helpful, although the use of heparin is controversial.

Warfarin-induced skin necrosis

Warfarin-induced skin necrosis is a rare condition that is due to microvascular thrombi provoked by a transient imbalance between procoagulant and anticoagulant factors. It most commonly affects the breasts, buttocks and thighs. It may occur on initiation of warfarin, especially in patients with heterozygous protein C or protein S deficiency because of the relatively short half-life of these proteins compared with prothrombin. It is also seen in association with heparin-induced thrombocytopenia. It is prevented by bridging the initiation of warfarin with heparin and avoiding high-loading doses. Treatment requires discontinuation of warfarin and starting therapeutic heparin. Intravenous infusions of protein C concentrate may also be used in the short term.

Haemostatic dysfunction associated with paraproteinaemia and amyloidosis

Paraproteinaemia

Bleeding and thrombotic complications both occur in association with paraproteinaemias. Abnormalities in laboratory tests are found much more frequently than clinical effects and these are being recognised more often, associated with increased surveillance. Bleeding is more common in Waldenström macroglobulinaemia and amyloidosis than multiple myeloma. Amongst myeloma patients, bleeding is most common in those with IgA paraproteinaemia. The circulating paraprotein in these conditions can: (i) cause hyperviscosity and lead to arterial and retinal bleeds due to abnormal wall shear stress; (ii) inhibit function or increase clearance of VWF/FVIII complex leading to acquired von Willebrand syndrome (aVWS); (iii) impair platelet aggregation; (iv) inhibit fibrin polymerisation and (v) have heparin-like anticoagulant function that can be reversed by protamine. Acquired VWS is most commonly seen with IgM paraproteins. Management of these conditions is directed towards the underlying cause and decreasing the paraprotein level. Plasma exchange is of help as a temporary measure. See below for management of aVWS.

Amyloidosis

The pathophysiology of bleeding associated with systemic amyloidosis is multifactorial. The type of amyloidosis and the pattern of organ involvement are important determinants of the haemorrhagic tendency (Chapter 29). AL amyloidosis, where liver and spleen involvement is frequent, is the commonest type associated with bleeding. FX deficiency is seen most commonly; however, decreased levels of all factors, including VWF, have been reported. The coagulation factor deficiencies are thought to be due to adsorption onto amyloid fibrils. Abnormal fibrin polymerisation and hyperfibrinolysis can also contribute to bleeding. Prolongation of the TT is the most common abnormality, seen in up to 90% of patients. A prolonged PT and APTT suggest FX deficiency. Abnormal coagulation screening tests are correct with normal plasma supporting the absence of a circulating inhibitor.

Platelet dysfunction may be seen, due to the binding of the amyloid light chains to the platelet membrane. Deposition of amyloid fibrils in the blood vessel wall and perivascular tissue may lead to impaired vasoconstriction and vessel fragility. This is exemplified by cerebral amyloid angiopathy, which can lead to intracerebral haemorrhage, especially in elderly non-hypertensive individuals. The microvascular involvement also explains the 'raccoon eyes' (bilateral periorbital purpura from coughing or prolonged inverted positioning for lower gastrointestinal procedures) and the 'pinch purpura' (skin pinching leading to purpura). Several treatments have been described, including factor replacement with FFP or PCCs, platelet transfusion, desmopressin and rFVIIa. Severe FX deficiency can be difficult to manage and first-line therapy is with PCCs or FFP although rapid clearance of FX may be seen.

Acquired inhibitors of coagulation factors

Inhibitory autoantibodies to all coagulation factors have been described, although those against FVIII and VWF are most common.

Acquired haemophilia A

Acquired haemophilia A has an annual incidence of about 1.5 per million and usually affects older patients with a median age of about 75 years. It affects males and females equally, except in younger patients where there is a female preponderance associated with pregnancy. Acquired haemophilia A presents with a typical bleeding pattern that is distinct from that seen in congenital haemophilia. Widespread subcutaneous bleeding is common, as are muscle bleeds and gastrointestinal and genitourinary bleeding. Neurovascular compression may be limb-threatening. Haemarthroses are relatively uncommon. Fatal bleeding, such as intracranial, pulmonary, gastrointestinal and retroperitoneal, occurs in about 3–8% of cases (Table 43.8). Patients remain at risk of severe bleeding until the inhibitor has been eradicated, even if they present with mild bleeding. Acquired haemophilia A is associated with an underlying autoimmune (systemic lupus erythematosus or rheumatoid arthritis), malignant, lymphoproliferative or dermatological (pemphigoid) disease or pregnancy in about half of cases (Table 43.9). If acquired haemophilia A

Table 43.8 Bleeding symptoms in patients with acquired haemophilia A*.

Site of bleeding	All bleeds at presentation (%)	Bleeds requiring treatment (%)
	N = 172	*N* = 65
Subcutaneous/skin	81	23
Muscle	45	32
Subcutaneous only	24	Not applicable
Gastrointestinal/ intra-abdominal	23	14
Genitourinary	9	18
Retroperitoneal/ thoracic	9	5
Other	9	23
Postoperative	0	11
Joint	7	2
None	4	Not applicable
Intracranial haemorrhage	3	0
Fatal	9	No data

* Patients had often had multiple bleeds.
(*Source:* Data from Collins *et al.* (2007) and [Morrison *et al. Blood* **81**: 1513–20 1993].)

Table 43.9 Diseases associated with acquired haemophilia A.

	Green and Lechner (1981)	Morrison and Ludlam (1993)	Collins *et al.* (2007)
Number of patients	215	65	150
Idiopathic (%)	46	55	63
Collagen, vascular and other autoimmune diseases (%)	18	17	17
Malignancy (%)	7	12	15
Skin diseases (%)	5	2	3
Possible drug reaction (%)	6	3	0
Pregnancy (%)	7	11	2

(*Source:* Data from [Green and Lechner, *Thrombosis and Haemostasis* **45**: 200–3, 1981], [Morrison *et al. Blood* **81**: 1513–20, 1993] and Collins *et al.* (2007).)

presents in association with pregnancy, it may recur in subsequent pregnancies and, if antenatal, the fetus may be affected due to passive transfer of antibody.

Early diagnosis and urgent treatment of bleeding are key to successful management. The diagnosis is suggested by the clinical presentation and an isolated prolonged APTT. FVIII inhibitors are time and temperature-dependent dependent and mixing studies with normal plasma must be incubated for 1–2 hours otherwise the diagnosis may be missed. The diagnosis is confirmed by a low FVIII and positive Bethesda titre (Figure 43.6). In some cases, diagnosis is complicated because all the intrinsic factors may appear to be low due to inhibition of FVIII in the factor-deficient plasma used to assay other intrinsic factors. Dilution will result in increased levels of the non-specifically reduced factors while FVIII remains low. It is important to exclude a lupus anticoagulant because this can also be associated with an apparently low FVIII and an APTT that does not correct with normal plasma, a chromogenic FVIII assay will not be affected by a lupus anticoagulant. Acquired FVIII inhibitors have complex kinetics so that not all FVIII is inhibited in a Bethesda assay. It is therefore often not possible to measure the inhibitor titre accurately. The FVIII level and inhibitor titre are not predictive of the severity of the bleeding but an undetectable level of FVIII and inhibitor above 20 BU means that the person is less likely to achieve a remission with steroids alone.

Treatment involves minimising invasive procedures, protecting the patient from trauma, treatment of bleeds and eradication of the inhibitor. At present, the available therapies to treat bleeds are with recombinant porcine FVIII or bypassing agents, namely rFVIIa or the activated PCC FEIBA (factor eight inhibitor bypassing activity). FEIBA and rFVIIa are equally efficacious. Multiple doses of bypassing agents may be required to control bleeds and prevent recurrence. There is a risk of thrombosis associated with bypassing agents, particularly in elderly patients. Human FVIII is unlikely to be efficacious in acquired haemophilia A, although mild bleeds in patients with low-titre inhibitors and measurable FVIII may respond to desmopressin. Recombinant porcine FVIII is efficacious if the anti-porcine titre is low, however, exposure to recombinant porcine FVIII is likely to lead to an increase in

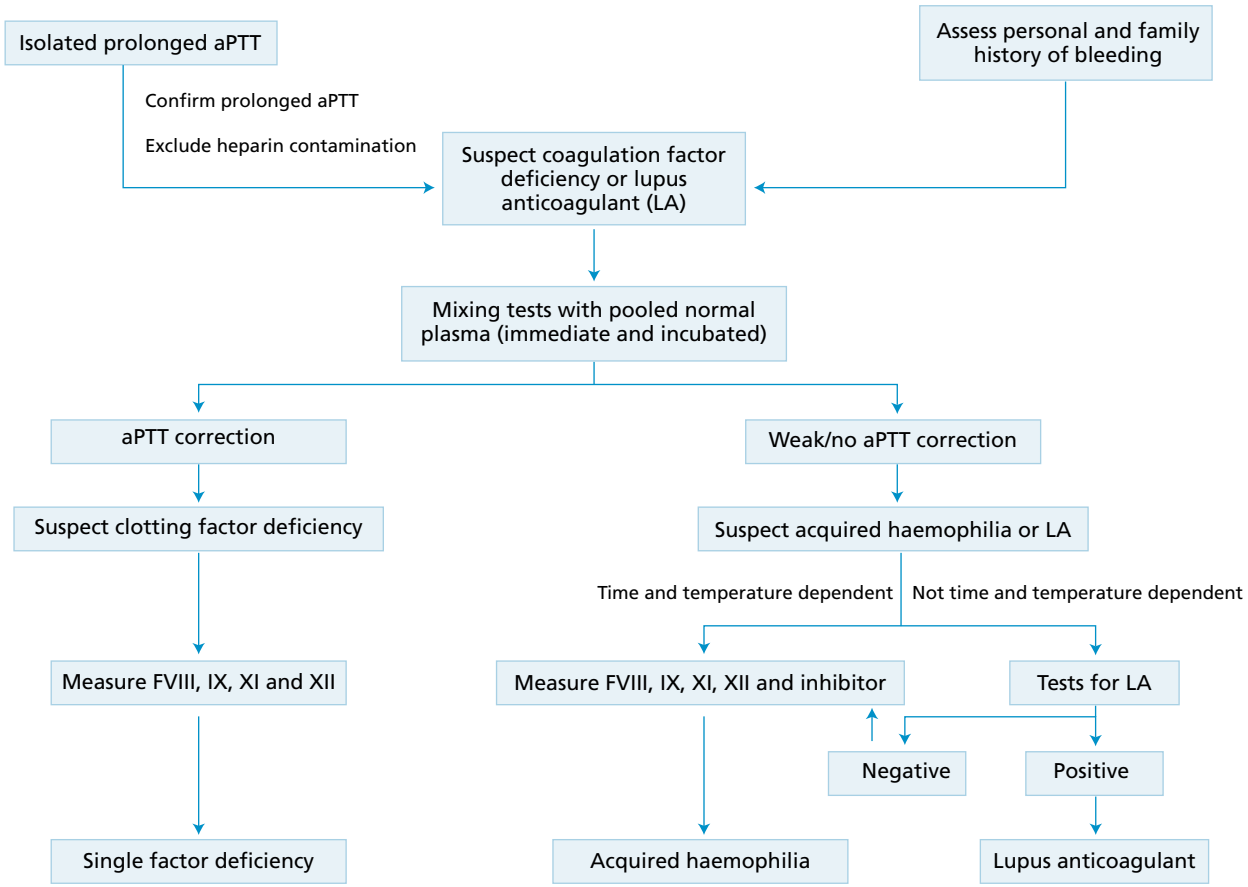

Figure 43.6 Diagnostic algorithm for an isolated prolonged APTT and possible acquired haemophilia A. A chromogenic FVIII level may be useful in complicated cases. Many centres proceed directly to factor assays and testing for a lupus anticoagulant rather than performing correction studies.

anti-porcine inhibitor titre. There are some case reports of the use of off-label Emicizumab although the safety of this treatment is unclear and it interferes with disease monitoring.

FVIII levels must be measured to monitor response to treatment and to decide on frequency and dose. If measureable levels can be achieved efficacy is good and this mode of treatment has not been associated with an increased risk of thrombosis. If standard haemostatic treatment fails, FVIII and immunoadsorption may be used, although this treatment modality is only available in a few specialised centres.

Immunosuppression should be started as soon as the diagnosis is made and is usually with steroids alone or steroids plus a cytotoxic agent such as cyclophosphamide or azathioprine, there are anecdotal reports of the use of mycophenolate. A response is usually seen after 2–3 weeks, but full remission takes a median of about 5 weeks. If first-line therapy fails, rituximab, ciclosporin or combinations of cytotoxic agents may be used. Intravenous immunoglobulin does not increase the response rate to other immunosuppressive agents.

Acquired von Willebrand syndrome

Acquired von Willebrand syndrome may be associated with an autoantibody, typically in the context of monoclonal gammopathy of undetermined significance, Waldenström macroglobulinaemia and other lymphoproliferative diseases and systemic lupus erythematosus. The antibody leads to either rapid clearance of VWF/FVIII or functional inhibition of VWF. Low VWF:Ag, VWF:RCo, FVIII and VWF:CB are found, and measurement of the VWF propeptide may be useful in demonstrating rapid clearance. *Ex vivo* mixing studies will not demonstrate an inhibitor if the mechanism is of increased clearance.

Low VWF levels are also seen in hypothyroidism and increased VWF is associated with hyperthyroidism. In myeloproliferative disorders high molecule weight VWF multimers may be consumed or degraded. Increased proteolysis of VWF in high-shear environments such as leaking cardiac values, extracorporeal circuits leads to a syndrome of acquired type 2A VWS. AVWS has also been reported in association with malignancy and it is suggested that it may be adsorbed onto malignant cells. Hydroxyethyl starches used as plasma expanders have been associated with bleeding secondary to VWF deficiency. Treatment options include desmopressin and VWF concentrates. High-dose intravenous immunoglobulin may increase the baseline VWF/FVIII and improve the recovery and half-life of infused VWF concentrate.

Acquired factor V deficiency

Acquired inhibitors to FV may arise spontaneously but are usually associated with exposure to topical thrombin preparations that have trace amount of bovine FV. Laboratory findings are of prolonged PT and APTT that do not correct with normal plasma. Patients may respond to FFP and, in resistant cases, platelets may be useful because they provide surface-associated FV which appears to bypass the inhibitor.

Acquired protein S deficiency

Autoantibodies to protein S have been reported in association with infection, particularly chickenpox. Patients present with skin necrosis and DIC.

Prothrombin deficiency associated with lupus anticoagulant

Although lupus anticoagulants are typically associated with thrombosis, occasionally cross-reactivity with prothrombin can lead to a bleeding disorder. Acquired deficiencies of other procoagulant and antifibrinolytic proteins have occasionally been described. Bleeding manifestations are variable and some patients with markedly abnormal laboratory tests do not bleed.

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CHAPTER 44

Congenital platelet disorders

44

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Introduction

A normal haemostatic system is able to provide a sequence of local events that culminates in the spontaneous arrest of bleeding from a traumatised blood vessel followed by sealing of any vascular discontinuity. Three closely related biological systems are involved: platelets, blood vessels and coagulation proteins. Platelets are anucleate cells that derive from cytoplasmic fragmentation of bone marrow megakaryocytes with a life span in the bloodstream of about 7–10 days. Platelets have a complex ultrastructure comprising a multitude of molecules that allow their adhesion and aggregation (a type of muscle-like contraction) and the malfunction of any of these may promote a specific disease (Figure 44.1). In the resting state, platelets normally do not interact with endothelial cells or other blood cells but do so when the vessel wall is disrupted. Adhesion to exposed subendothelial components allows platelet activation, aggregation and release of active substances from intracellular organelles such as adenosine diphosphate (ADP) that amplify and propagate platelet activation and aggregation into a plug that fills in any voids in a severed blood vessel. During this primary haemostasis phase, platelets transiently stop the bleeding from damaged blood vessels and provide a surface onto which plasma fibrinogen starts to polymerise into a fibrin mesh that strengthens the platelet plug, the so-called secondary haemostasis phase. Clinically, abnormalities of platelet adhesion and aggregation present with a history of spontaneous and/or easy bruising and a prolonged skin bleeding time or abnormal closure time on the Platelet Function Analyser (PF100). Abnormal bleeding due to defective primary haemostasis, that is, numerical or functional platelet defects, shows characteristic features distinct from those seen in disorders of secondary haemostasis, that is, plasma coagulation factors defects (Table 44.1). The occurrence of superficial bleeding in patients with platelet disorders as opposed to deeper bleeding in patients with clotting factor disorders is a useful clinical point, but it must be remembered that platelets and coagulation activation occurs almost simultaneously. Bleeding due to platelet abnormalities manifests as haemorrhages from small vessels: petechiae usually develop on the skin and the visible mucous membranes, but they may be distributed throughout the body, including internal organs. Characteristically, bleeding resulting from platelet disorders is immediate and transient, tends to stop promptly with local pressure and does not recur when the pressure is removed. When this pattern of bleeding occurs in the neonatal period, in infancy and in childhood, a congenital platelet disorder should be suspected. However, the disease may be clinically silent and the patient may enter adulthood without incurring in any bleeding episodes.

The family history is of major importance, providing a characteristic pattern of inheritance, but it should be remembered that a negative family history does not exclude an inherited platelet abnormality; in fact, the family history is usually negative in autosomal recessive traits. A comprehensive medical history and a careful clinical examination of the patient presenting with a haemorrhagic disorder are crucial for the correct diagnosis: the garnered information may dictate the choice of laboratory tests and together they may lead to the diagnosis, when possible, of the platelet abnormality. Congenital platelet defects may give rise to bleeding syndromes of varying severities and

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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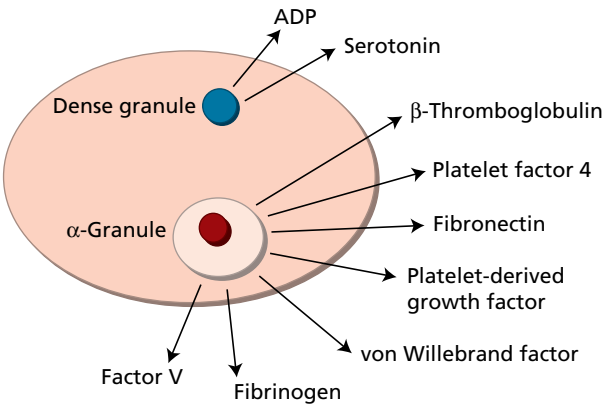

Figure 44.1 Schematic representation of the complex structure of platelets. Abnormalities of any platelet complex can lead to an alteration of a specific function.

are difficult to classify because of the rarity of several forms, the heterogeneity and the incomplete knowledge about many of them. Table 44.2 classifies congenital platelet disorders into two main groups: thrombocytopenias and thrombocytopathies. Each group is further divided according to specific criteria based on functional and biochemical defects. Such a classification, as others, should be viewed as tentative, because some disorders may be characterised by multiple pathogenetic factors, whereas some others are grouped together for convenience of classification rather than on their known physiopathology.

Thrombocytopenias

Thrombocytopenia defines a subnormal number of platelets in the peripheral blood, usually below 150×10^9L. Acute thrombocytopenia is the most frequent cause of severe bleeding and the risk of haemorrhage is inversely proportional to the platelet count, with spontaneous bleeding occurring more frequently at a platelet count below 20×10^9L. When automated methods are used, pseudo-thrombocytopenia can be observed. Different mechanisms can cause a false low platelet reading, and this phenomenon may occur in a wide variety of clinical disorders. Non-technical factors inducing pseudo-thrombocytopenia include paraproteins, cold agglutinins, giant platelets, previous contact of platelets with foreign surfaces (i.e. dialysis membrane), lipaemia and EDTA. The possibility of a pseudo-thrombocytopenia must be ruled out through manual counting and/or examination of an adequately stained blood film before concluding that a patient has true thrombocytopenia.

Table 44.1 Main specific clinical differences between diseases of coagulation factors and platelet disorders.

Findings	Disorders of coagulation	Platelets/vessels
Onset of bleeding	Delayed after trauma	Spontaneous or immediately after trauma
Mucosal bleeding	Rare	Common
Petechiae	Rare	Characteristic
Deep haematomas	Characteristic	Rare
Ecchymoses	Large and solitary	Small and multiple
Hemarthrosis	Characteristic	Rare
Bleeding from superficial cuts and scratches	Minimal	Persistent; often profuse
Sex of patient	80–90% male	Equal

Table 44.2 Classification of congenital platelet disorders.

a	Thrombocytopenia	
1	Non-inherited thrombocytopenia	Drugs and chemical agents
		Isoimmune throbocytopenia
		Infiltration of bone marrow
		Infections
		Other causes
2	Inherited thrombocytopenias	Thrombocytopenias with reduced platelet size
		Thrombocytopenias with normal platelet size
		Thrombocytopenias with increased platelet size
b	Trombocytopathies	
1	Disorders of platelet adhesion functions	
2	Disorders of platelet signalling transduction functions	
3	Disorders of platelet aggregation functions	

Non-inherited congenital thrombocytopenia

Congenital thrombocytopenia not ascribed to inherited causes may be the result of different pathogenetic mechanisms: most likely it is due to deficient marrow platelet production or enhanced platelet destruction.

Alloimmune thrombocytopenia

Immune thrombocytopenia may also result from the placental transfer of platelet antibodies formed as the result of active immunisation of the mother by fetal platelet isoantigens if the fetus has inherited a paternal platelet-specific antigen that induces antibody formation (see also Chapter 54). Transplacental passage of the maternal antibody, usually of the IgG isotype, may induce severe fetal thrombocytopenia. This usually occurs when the mother has PlA1-negative platelets

and the fetus carries PlA1-positive platelets. Newborns may show severe petechiae, purpura and/or bleeding at the time of birth; alternatively, they may appear normal at delivery and manifest severe bleeding within the first postnatal week. In this case, thrombocytopenia usually improves within 1 month, but severe and fatal intracranial haemorrhages may occur.

Drugs and chemical agents

The maternal use of drugs and chemical agents during pregnancy may cause thrombocytopenia by suppressing platelet production, by damaging platelets directly or by inducing the formation of platelet antibodies. Maternal ingestion of ethanol, thiazides, chlorpropamide, tolbutamide, oestrogens, steroids and other drugs may selectively suppress thrombopoiesis. Alkylating agents, antimetabolites or chemotherapeutic drugs capable of crossing the placenta may induce predictable marrow suppression leading to severe thrombocytopenia in newborns. The maternal ingestion of quinine, quinidine, hydralazine, or antibiotics known to induce immune thrombocytopenia may induce congenital immune thrombocytopenia. In these newborns, thrombocytopenia usually improves rapidly, and it is unlikely to cause severe or fatal haemorrhages.

Bone marrow infiltration

Congenital thrombocytopenia caused by bone marrow infiltration is extremely rare, being limited to cases of disseminated reticuloendotheliosis and congenital leukaemia. Thrombocytopenia with or without associated myeloid and erythroid depression occurs in children with numerous infiltrative disorders, including solid tumours, Gaucher's disease, Niemann–Pick disease and the mucopolysaccharidoses.

Infection

Thrombocytopenia, usually mild, but at times very severe, is commonly seen in infected new-born: several mechanisms are probably responsible, indeed viral infections may directly impair platelet production as a result of direct megakaryocyte invasion, while the formation of antigen–antibody complexes in response to the viral infection indirectly allows the sequestration of platelets by the reticuloendothelial system. Maternal infection with toxoplasma, cytomegalovirus, rubella, herpes viruses or hepatitis varicella, as well as recent maternal vaccinations (rubella), may induce congenital thrombocytopenia.

Other causes

Children born to women with chronic idiopathic thrombocytopenic purpura may develop congenital thrombocytopenia due to maternal autoantibodies crossing the placenta and binding to fetal platelets. Other causes of congenital thrombocytopenia secondary to increased platelet consumption or destruction include common maternal disorders (pre-eclampsia, systemic lupus erythematosus or other autoimmune diseases, particularly if the mother has antiphospholipid antibodies). Finally, a moderate to severe thrombocytopenia has been described in giant cavernous haemangioma, where platelets are consumed primarily within the tumour itself and the severity tends to parallel the size of the vascular tumour, whether or not in association with a degree of disseminated intravascular coagulation.

Inherited thrombocytopenias

Several inherited diseases may manifest with thrombocytopenia, as an accompanying feature of a generalised bone marrow failure (Fanconi's anaemia) or of metabolic diseases causing marrow infiltration (Gaucher's disease). These and other complex clinical syndromes (i.e. Noonan's syndrome, CATCH 22 syndrome, etc.) will not be discussed in this chapter. Inherited thrombocytopenias are very rare. In some instances, thrombocytopathy may coexist with thrombocytopenia (i.e. Bernard–Soulier syndrome, Wiskott–Aldrich syndrome, etc.). These conditions will be discussed in the appropriate section according to the most prominent defect. Helpful criteria to classify inherited thrombocytopenias are platelet size and the presence (syndromic) or the absence (non-syndromic) of other clinical features.

Inherited thrombocytopenias with reduced platelet size

The Wiskott–Aldrich syndrome (WAS) is a rare (1/250,000) X-linked recessive disorder characterised by eczema, susceptibility to infections due to defective cellular and humoral immunity, and thrombocytopenia with reduced platelet size. The gene responsible for WAS maps to Xp11.1 and codes for a protein, WASp, expressed only in haemopoietic-derived cells and involved in the transduction of the signals from the cell surface receptor to the actin cytoskeleton. Intermittent bleedings, recurrent bacterial and viral infections and progressive eczema develop during the first months of life. Death at an early age commonly results from intracranial haemorrhage, infection or lymphoreticular malignancy. A number of kindreds have been reported in whom X-linked thrombocytopenia (XLT) occurred alone or in association with partial manifestations of WAS; indeed, a pathogenic variant within the WAS gene is also responsible for XLT (X-linked thrombocytopenia); affected patients suffer mainly from isolated bleeding episodes. Pathogenic variants in the *FYB* gene coding for adhesion and degranulation-promoting adaptor protein lead to impaired cytoskeleton remodelling that prevents full megakaryocyte maturation, leading to thrombocytopenia. Biallelic-null pathogenic variants in the *PTPRJ* gene

coding for tyrosine phosphatase also impairs megakaryocyte maturation, leading to thrombocytopenia as well as platelet dysfunction.

Inherited thrombocytopenias with normal platelet size

Congenital lack of megakaryocytes is a rare form of thrombocytopenic purpura in the newborn and may occur with skeletal, renal or cardiac malformations. Isolated congenital amegakaryocytic thrombocytopenia (CAMT) is an autosomal recessive syndrome leading to bone marrow aplasia later in childhood. This disorder is associated with abnormalities in the expression or function of the thrombopoietin receptor c-MPL, of which a series of pathogenic variants have been identified. Skeletal anomalies are often associated such as bilateral agenesis of the radius (thrombocytopenia with absent radii (TAR) syndrome). The TAR syndrome is an autosomal recessive disease characterised by severe, even fatal, haemorrhagic manifestations. The molecular basis of the syndrome is due to alterations of the *RBM8A* gene and often involves a microdeletion on chromosome 1q21.1. In some infants, the ulnae and humeri may also be absent alongside other skeletal abnormalities. Proximal radioulnar synostosis, syndactyly and other skeletal abnormalities are reported in association with an autosomal dominant congenital amegakaryocytic thrombocytopenia in infants carrying heterozygous pathogenic variants of the *HOXA11* gene. Less commonly, children present with cardiac and other minor defects. An autosomal dominant, though moderate form of thrombocytopenia with a mild or absent bleeding tendency has been associated with pathogenic variants in the *ANKRD26* gene (ANKRD26-related thrombocytopenia) and with haematological malignancies, in particular acute myeloid leukaemia. The Schulman–Upshaw syndrome, caused by pathogenic variants in the *ADAMTS13* gene, is characterised by a neonatal thrombotic thrombocytopenic purpura (congenital microangiopathic haemolytic anaemia) that relapses frequently but is responsive to fresh frozen plasma infusion. Rare pathogenic variants in genes that regulate different steps of megakaryocyte maturation may give rise to thrombocytopenia with normal size: a pathogenic variant in the mitochondrial protein CYCS involving apoptosis may induce immature proplatelet formation; pathogenic variants in SRC, a kinase that regulates several signalling pathways and in IKZF5, a transcriptional regulator, may disrupt megakaryopoiesis by a so far unknown mechanism.

Inherited thrombocytopenias with increased platelet size

Thrombocytopenias with increased platelet size is characterised by increased platelet volume (macrothrombocytopenia) and reduced platelet numbers, and represent the commonest inherited thrombocytopenias. Many of them have been characterised at the molecular level, whereas others still await a clear identification of the molecular defect. Giant platelets and moderate thrombocytopenia are most frequently found in certain populations of Mediterranean origin and also may be associated with other inherited or congenital syndromes, such as MYH9-related disease, Bernard–Soulier syndrome, stomatocytosis, etc.

MYH9-related disease is a rare autosomal dominant disease characterised by the presence of thrombocytopenia with giant platelets and basophilic inclusions (Dohle's bodies) within granulocytes, and despite mild neutropenia, there is no increased susceptibility to infection. About 50% of patients present significant thrombocytopenia deriving from an ineffective thrombopoiesis, but despite the impairment of platelet shape change, life-threatening haemorrhage is rare. The platelets of patients suffering from MYH9-related disease not only show a volume that can be twice as normal, but also display bizarre morphology and hyper-granularity. The condition includes a group of autosomal dominant diseases, known in the past as May–Hegglin anomaly and Sebastian, Fetchner and Epstein syndrome, according to the presence of hearing loss, glomerulonephritis and cataract. Subsequently, because of the high variability of the clinical phenotype, even in members of the same kindred, these eponyms have been abandoned and the term MYH9-related disease has been proposed independently of the presence of Dohle-like bodies and non-haematologic features. The molecular basis of these syndromes has been elucidated. All these clinical entities are caused by pathogenic variants that occur within a gene (*MYH9*) located on the long arm of the chromosome 22 (22q12-13). This gene encodes for the heavy chain of non-muscle myosin IIA (NMMHC-IIA), a protein involved in the contractile activity of the platelet cytoskeleton. On the whole, all diseases may be grouped as MYH9-related disease (Table 44.3).

The Grey Platelet syndrome (GPS) is a rare disorder inherited as an autosomal recessive trait due to pathogenic variants within the *NBEAL2* gene, although a dominant transmission has been also shown, characterised by large platelets with a selective deficiency in the number and content of α-granules (see also in disorders of platelet aggregation). Because of this, platelets are either markedly hypogranular or agranular and have subnormal levels of α-granule proteins, such as fibrinogen, von Willebrand factor, thrombospondin, β-thromboglobulin and platelet factor 4. A missense pathogenic variant at position 759 in the *GATA-1* gene, inducing an amino-acid change (Arg216Gln), which segregates in a X-linked GPS, has been recently identified, suggesting that the *GATA-1* gene is required for platelet α-granule regulation. Thrombocytopenia is usually pronounced and severe bleeding may occur.

Mediterranean macrothrombocytopenia is an asymptomatic disorder with moderate isolated thrombocytopenia and large platelets inherited as an autosomal dominant trait. The condition is characterised by mild or no clinical manifestations and

Table 44.3 Main clinical differences amongst syndromes due to pathogenic variants within the *MYH9* gene.

	Macro	Renal disease	Hearing loss	Cataract	Leukocyte
Disease	thrombocytopenia				Inclusion*
MHA	Present	Absent	Absent	Absent	Present
SBS	Present	Absent	Absent	Absent	Present
EPS	Present	Present	Present	Absent	Absent
FTS	Present	Present	Present	Present	Present

* Ultrastructure differences in leukocyte inclusions exist (see Balduini *et al*. 2003).
MHA, May-Hegglin anomaly; SBS, Sebastian's syndrome; EPS, Epstein's syndrome; FTS, Fetchner's syndrome.

normal bone marrow megakaryocytes, platelet survival and *in vitro* platelet functions. Platelets from some patients suffering from Mediterranean macrothrombocytopenia show a reduced expression of the GPIb-IX platelet receptor, and heterozygous pathogenic variants within the *GPIbα* or *Ibβ* genes have been described. These patients with Mediterranean macrothrombocytopenia may be viewed as carrying a heterozygous form of Bernard–Soulier syndrome (see below). The remaining patients with Mediterranean macrothrombocytopenia do not show a reduction of the content of the GPIb-IX platelet receptor and the pathogenesis of this form needs further clarification.

Type 2B von Willebrand disease is a thrombocytopenia resulting from enhanced platelet destruction. Patients suffering from this syndrome show a qualitative abnormality of plasma von Willebrand factor (VWF): pathogenic variants within exon 28 of the *VWF* gene yields a VWF with an increased affinity for the platelet receptor GPIb-IX allowing for faster clearance of the resulting VWF-platelet complexes that leads to thrombocytopenia and to the selective loss of the largest VWF multimers from plasma. In general, the degree of thrombocytopenia is moderate and the bleeding is of variable severity. A *VWF* gene pathogenic variant was identified in a Montreal kindred, previously labelled as Montreal platelet syndrome, a very rare platelet disorder characterised by giant platelets, reduced platelet count and spontaneous *in vitro* platelet aggregation: this kindred has now been reclassified as type 2B von Willebrand disease. Type 2B von Willebrand disease should be distinguished from the rare pseudo-von-Willebrand disease (or platelet-type von Willebrand disease). The defect in this autosomal dominant condition results from gain of function pathogenic variants in the gene encoding for the GPIb-subunit of the platelet receptor, increasing the affinity for VWF resulting in spontaneous binding of platelets to VWF with shortened platelet survival and thrombocytopenia. The reasons why most patients suffering from platelet-type von Willebrand disease show variable enlarged platelets remain unclear.

Jacobsen and Paris–Troussau syndromes are other very uncommon causes of macrothrombocytopenia. The Jacobsen syndrome is a contiguous gene syndrome inherited as an autosomal dominant trait characterised by a chromosomal deletion encompassing the long arm of the chromosome 11 (11q23.3–11q24.2). Paris–Trousseau syndrome identifies patients who present with macrothrombocytopenia, abnormal platelet function and morphologically abnormal megakaryocytes in the bone marrow. A heterozygous deletion of the *FLI1* gene has been described in unrelated children with deletions of 11q23 and Paris–Trousseau thrombocytopenia. Interestingly, in patients suffering from Jacobsen syndrome without thrombocytopenia, the chromosomal deletion did not include the *FLI1* gene, suggesting that deletion of this gene is specific for the thrombocytopenia that occurs in most patients with Jacobsen and Paris–Trousseau syndromes.

Pathogenic variants in GFI1b, a transcriptional factor involved in haematopoietic stem cells homeostasis and megakaryocyte maturation leads to thrombocytopenia, as well as defects of the Mg^{2+} channel TRPM7, a possible modulator of cell survival, that causes alterations in the cytoskeleton preventing proplatelet formation. In this respect, the cytoskeleton remodelling that leads to megakaryocyte maturation and proplatelet formation is under the control of several genes: *DIAPH1, ACTN-1, TUBB-1*, that code for tubulin beta 1, a major component of microtubules, *TPM4*, that codes for tropomyosin 4, FLNA that codes for filamin A: pathogenic variants in any of these lead to defective proplatelet formation hence thrombocytopenia.

Thrombocytopathies

Platelets participate in haemostasis by adhering to exposed elements of the subendothelial matrix. Once adhered onto the subendothelial surface they activate and secrete the proteins contained in their granules and aggregate with each other. Abnormalities in any of these stages, adhesion, activation, secretion and aggregation may give rise to a functional inherited platelet disorder. Patients suffering from any of these

diseases usually show a bleeding diathesis with a prolonged bleeding time, abnormal PF100 closure times in the presence of a normal platelet count. The most accurate test for evaluating platelet activation is light transmission aggregometry, which uses platelet-rich plasma or washed platelets, and monitors the change in light transmission upon agonist addition and simultaneously dense granule secretion. However, diagnosing a platelet function defect can be difficult, because of need for specific expertise, and skilled investigators are required. A further major challenge is the wide overlap with the response of healthy subjects. Up to 40% of patients presenting with a platelet function defect, light transmission aggregometry fails to correctly identify the thrombocytopathy.

Disorders of platelet adhesion

The interaction of platelet receptors with blood components, collagen and fibronectin in the sub-endothelium layer allows platelet adhesion to the same, aided by the bridging effect of VWF (Figure 40.2). The platelet surface is equipped with a series of receptors that interact with one or more of the previous sub-endothelial factors. The most important receptors are the GPIb-IX and the α2-β1 (previously known as GPIa-IIa). The α2-β1 is a member of the integrin β-1 subfamily that binds collagen alongside GPIV and GPVI. Sequential and multiple receptor-ligand binding reinforces adhesion and provides the simultaneous stimuli that are required to mobilise intracellular calcium and induce protein phosphorylation. Bernard–Soulier Syndrome (BSS) is a bleeding disorder characterised by giant platelets, mild or moderate thrombocytopenia and prolongation of the skin bleeding time of PF100 closure times, disproportionate to the degree of thrombocytopenia. BSS is a recessively inherited autosomal disorder due to abnormalities of the GP Ib-IX-V receptor complex and consanguinity is common in reported kindreds. The frequency of BSS has been estimated to be approximately 1 case in one million people. Bleeding may be severe and fatal haemorrhages may occur. Cutaneous bleeding, muscular and internal organ haemorrhages are common. Epistaxis and menorrhagia may be difficult to control, and hemarthroses have also been reported. Platelet counts range from as low as 20×10^9/L to near normal and, on the peripheral blood film, over 80% of the platelets are usually larger than 2.5 µm, often up to 8.0 µm, in diameter. The number of bone marrow megakaryocytes is usually normal. Patients presenting with BSS show an absent platelet agglutination in response to ristocetin (in the presence of human VWF), normal aggregation, ATP secretion and thromboxane (TX) B_2 formation in response to a variety of aggregating agents, and delayed response to thrombin. Because of the defective binding to VWF, platelets in BSS have substantial reduction in their ability to adhere to sites of vascular injury where subendothelial VWF becomes exposed. As a consequence, the primary hemostatic response, plug formation, is impaired, and prolonged bleeding occurs. Variable levels of the glycoprotein (GP) Ib-IX-V complex have been detected on the platelet surface of patients with BSS, some patients exhibiting near normal GP Ib-IX-V amounts (variant type of BSS). In spite of the differences in glycoprotein content, clinical bleeding and platelet functional and morphologic abnormalities, these patients are indistinguishable from the classical BBS phenotype. The entire cDNA encoding the protein chains comprising the GP Ib-IX-V receptor complex has been sequenced, clarifying the molecular basis of the syndrome. This complex consists of four proteins: the disulfide-linked α- (135 kD) and β-chains (25 kD) of GPIb, and the non-covalently associated subunits GPIX (22 kD) and GPV (82 kD). They all share structural and functional features suggesting a common evolutionary origin. Different transcripts encode the four polypeptide chains and, with the exception of that of GPIbβ, genes show continuous (intron-depleted) open reading frames. In addition, each element contains one or more homologous 24-amino-acid leucine-rich glycoprotein repeats. The genetic heterogeneity in the glycoprotein content of BSS patients shows that multiple molecular abnormalities may lead to a similar clinical disorder and implies that BSS may be the result of defects within the subunits that hamper the coordinated expression of the complex on the platelet membrane. In this respect, BSS would resemble abnormalities of other multi-subunit complexes, in which a defect in a single subunit prevents the assembly and the surface expression of the other sub-units and

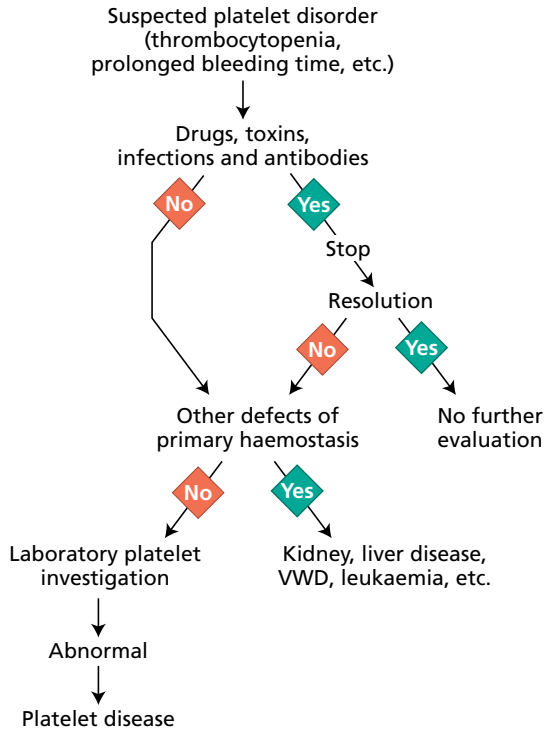

Figure 44.2 Flowchart for the investigation of a suspected platelet disorder.

therefore of the full complex. In addition to quantitative and qualitative abnormalities of the *GPIbα* gene, the recognised BSS phenotype has also been documented in patients with detrimental pathogenic variants within platelet *GPIX* and *GPIbβ* genes.

A defect of one of the platelet collagen receptors has been described in very few patients. These patients showed mild bleeding disorders and a selective but varied impairment to collagen induced adhesion to subendothelial surfaces and to collagen-induced platelet aggregation. Platelets have two major receptors for collagen; α2β1 integrin, with a major role in adhesion of platelets to subendothelial surfaces, and GPVI, mainly involved in platelet activation. Thus, it is conceivable that collagen binding to platelets is a multistep mechanism, involving first the binding of platelets to exposed collagen at the flowing blood/sub-endothelium interface by means of the α2β1 receptor and then platelet activation through a second receptor GPVI.

Disorders of platelet signalling transduction

Once adhered to the subendothelial factors, the platelet membrane transduces to the intracellular environment the signals required for platelet activation, such as calcium mobilisation, protein phosphorylation and the generation of arachidonic acid (AA). Activated platelets release the molecules stored in their granules, some of which aid in the recruitment of additional platelets and lead to the formation of the platelet plug typical of primary haemostasis (Figure 44.1). The signalling events involved in platelet activation, aggregation and secretion are multiple. Any abnormalities in platelet signalling may underlie a platelet dysfunction. The term platelet signalling disorders defines a group of heterogeneous abnormalities in signal transduction and platelet secretion. Congenital defects of platelet signalling mechanisms are put together for convenience of classification rather than on the basis of knowledge in the pathophysiology of specific diseases. Patients suffering from these defects represent the vast majority of subjects presenting with inherited thrombocytopathies. Many platelet receptors are coupled to G-proteins. P2Y1 and receptors for thromboxane A2, and thrombin act via Gαq. Receptors for prostaglandins activate, whereas P2Y12 inhibits adenylate cyclase through Gs and Gi2, respectively. Platelet activation by means of binding to receptors gives rise to hydrolysis of phosphoinositide by phospholipase C, leading to the formation of inositol triphosphate, which in turn, functions as a messenger to release calcium from intracellular stores. In addition, hydrolysis of phosphoinositide leads to the formation of diacylglycerol, which activates protein kinase C. The activation of the protein kinase C is thought to play a major role in platelet secretion and in the activation of the αIIb-β3 complex. Although defects in phospholipase C activation, calcium mobilisation and protein phosphorylation have been suggested in several patients, few patients have been reported with deficiency in platelet Gαq, Gs hyperfunction and reduced expression of phospholipase C-β2. Following stimulation, platelet phospholipase A2 mobilises the AA from the phospholipid pool. Then, the AA is metabolised by cyclo-oxygenase and thromboxane synthase to form thromboxane A2, a strong platelet-aggregating agent, which is necessary for a secretion response. A defect in AA mobilisation and thromboxane A2 production has been identified in some patients. Individuals with this defect showed an abnormal aggregation and secretion in response to a series of stimulating factors, but a normal production of thromboxane A2 in response to AA. Several patients presenting on the clinical side with a variable bleeding diathesis and on the laboratory side with a slightly prolonged bleeding time and an impaired platelet aggregation were diagnosed with a deficiency of cyclo-oxygenase and a defect in thromboxane A2 formation.

Disorders of platelet aggregation

Circulating epinephrine, ADP and collagen at the site of vessel injury, as well as thrombin deriving from coagulation activation, are the exogenous platelet agonists that promote an increase of the intracytoplasmic platelet concentration of Ca^{2+} followed by the release of the content of the α and δ granules alongside the generation of thromboxane A_2, all endogenous platelet agonists. Whether exogenous or endogenous, all platelet agonists, induce the inside-out expression of membrane GPIIb/IIIa, the fibrinogen receptor: the binding of soluble fibrinogen to GPIIa/IIIb favours platelet-platelet interaction and allows increased strength in the resulting aggregate.

Accordingly, the lack of GPIIb/IIIa expression represents the most severe platelet aggregation disorder whereas the selective impairment of platelet receptors (ADP, epinephrine, thromboxane and collagen) and storage pool disease represent less severe platelet function defects. To discriminate amongst disorders of these pathways, a series of substances are employed to challenge platelets *in vitro* and the manner they respond to these stimuli helps to identify specific thrombocytopathies (Table 44.4).

Glanzmann's thrombasthenia (GT) is a bleeding diathesis marked by a prolonged bleeding time, a normal platelet count and the absence of aggregation in response to ADP, collagen, arachidonic acid and thrombin. Platelet agglutination induced by ristocetin and VWF is normal. This congenital bleeding disorder is associated with an impaired or absent clot retraction. GT is one of the less common congenital bleeding disorders (prevalence 1/1,000,000) and is transmitted as an autosomal recessive trait with consanguinity reported in affected kindreds. The clinical features are those expected with platelet dysfunction: easy and spontaneous bruising, mucosal membrane bleeding, subcutaneous haematomas and petechiae; occasionally. Patients suffer from intra-articular bleeding causing hemarthroses and fatal haemorrhages have been described. Quantitative or qualitative (variant GT forms) abnormalities of

Table 44.4 Platelet response to aggregating agents in different thrombocytopathies.

	ADP	Epinephrine	Collagen	Arachidonic	Ristocetin
Disorder				Acid	
Bernard-Soulier	Normal	Normal	Normal	Normal	Absent
Pseudo-vWD	Normal	Normal	Normal	Normal	Increased at low doses
ADP receptor defect	Impaired	Impaired	Impaired	Impaired	Present
Epinephrine receptor defect	Normal	Impaired	Normal	Normal	Present
Collagen receptor defect	Normal	Normal	Impaired	Normal	Present
Defect of signal transduction	Variable impairment	Variable impairment	Variable impairment	Variable impairment	Present
Glanzmann's thrombasthenia	Absent	Absent	Absent	Absent	Present
δ-SPD	Impaired	Impaired	Impaired	Variable	Present
Thromboxane receptor defect	Impaired	Impaired	Impaired	Impaired	Present

the platelet αIIbβ3 integrin (previously known as the glycoprotein complex IIb/IIIa) have been shown to be responsible for this disorder. αIIb and β3 subunits are prominent integral components of the platelet membrane that form heterodimers containing specific sites for platelet-to-platelet cohesion. The αIIbβ3 integrin serves as platelet receptor for fibrinogen, fibronectin, vitronectin and VWF. In addition, the $\alpha IIb\beta_3$ integrin modulates, to some extent, calcium influx, cytoplasmic alkalinisation, tyrosine kinase phosphorylation and clot retraction. Clinical heterogeneity of GT has been assessed on the basis of platelet function testing or using crossed immune-electrophoresis, Western blot, flow cytometry and fibrinogen binding. Type I GT is characterised by the lack of surface-detectable αIIbβ3 complex and a profound defect in platelet aggregation and clot retraction. Type II GT is characterised by markedly reduced, amounts of the αIIbβ3 receptor on the surface, usually from 10 up to 20% of normal values. Therefore, platelets have enough receptors to allow the formation of micro aggregates but are still profoundly unable to form large aggregates. The clot retraction is only moderately impaired. In addition, a series of patients presented with a variant form with almost normal levels of the αIIbβ3 complex, which is dysfunctional in that platelets, when activated, can neither aggregate nor bind fibrinogen. An extreme variability in the clinical symptoms is present, even amongst patients with similar degrees of platelet abnormality and prolongation of the bleeding time. In general, no aggregation abnormalities are detected in heterozygotes, but a decreased amount of the αIIbβ3 integrin has been reported, platelet content being approximately one-half of the normal amount.

A pathogenic variant in the *FERMT3* gene is responsible for the recessive leucocyte adhesion deficiency type-III; the impairment of the actin function within the cytoskeleton prevents the proper inside-out GpIIb/IIIa activation leading to a GT phenotype. A pathogenic variant within *RASGRP2*, the gene coding for a nucleotide exchange factor in the monomeric GTPase Rap1 conducive to the inside-out signalling pathway of the GP IIb/IIIa, prevents the GTPase to respond to the increase in Ca^{2+} following platelet stimulation, hence the pathogenic variant is associated with reduced platelet response to multiple activators.

Defects of platelet ADP receptors have been characterised in a few patients and all suffered from a bleeding diathesis. On the platelet surface, different types of ADP receptors have been identified, two G-protein-coupled receptors, P2Y1 and P2Y12. The P2Y1 receptor is responsible for the shape change of platelets and transient platelet aggregation, giving rise to centralisation of platelet granules and formation of filopodia, while the P2Y12 receptor is involved in the amplification of the response and in the stabilisation of platelet aggregates through the full activation of the αIIbβ3 integrin. The P2Y12 receptor defect is inherited as an autosomal recessive trait and most patients so far identified were born from consanguineous parents. In these patients, a blunted platelet aggregation in response to ADP, with a retained shape change, has been reported. Only one patient has been briefly reported with a defect of the P2Y1 receptor. This patient showed impaired platelet aggregation in response to ADP and other agonists The selective impairment of epinephrine receptors, α2 adrenergic receptors (α2AR), has been associated with bleeding. A number of individuals with an impaired aggregation response to epinephrine and a congenital defect of platelet α2AR receptors have been described, and some of them presented a history of easy bruising. A selective impairment of the thromboxane A2 receptor has been described in several patients with a mild lifelong disorder, consisting of mucosal bleeding and easy bruising, and a defective platelet aggregation in response to several agents, but not thrombin. An autosomal dominant inheritance has been suggested in some cases.

Defects of endogenous agonists are more frequent than GT and the most common are the storage pool deficiency (SPD) syndromes that may be classified according to the decreases of the content of α-granules, dense (δ) granules or both (Figure 44.1). Partial or total deficiency of α-granules with normal amounts of δ granules typify the GPS.

The GPS is caused by a homozygous or compound heterozygous pathogenic variant in the neurobeachin-like 2 (*NBEAL2*) gene that codes for the NBEAL2-protein, member of a family of proteins that includes also the Beige and Chediak-Higashi (BEACH) domains involved in vesicle trafficking that is critical for the development of α granules. The GPS is autosomal recessive, affected people suffer mucocutaneous bleeding in the presence of moderate thrombocytopenia and large and pale platelets are seen on blood film. The syndrome is progressive, in that thrombocytopenia can worsen towards adolescence and adulthood, accompanied by a degree of myelofibrosis and occasionally splenomegaly. Additional genes (*GATA1, VPS33B, VIPAS39, GFI1B* and *PLAU*) are involved in congenital α-granule deficiency, but it is not distinguishable whether α-granule deficiencies are a single phenomenon of unknown genetic cause or as a component of multisystemic diseases involving all lysosome-like organelles.

Deficiency of δ granules typify Hermansky–Pudlak syndrome (HPS) and Chediak´-Higashi syndrome (CHS). HPS is characterised by oculocutaneous albinism, the absence of platelet dense granules and infiltration of ceroid-pigmented reticuloendothelial cells in the lung and the colon, leading to pulmonary fibrosis and inflammatory bowel disease. The causative genes encode dysfunctional proteins that impair the trafficking of lysosomes and vesicles. The genetic heterogeneity of HPS is quite wide and so far, pathogenic variants in several orthologous genes associated with different protein-associated complexes (HPACs) have been found in HPS patients: *BLOC1S3, BLOC1S5, BLOC1S6, DTNBP1* (HPAC BLOC-1 Deficiency); *HPS3, HPS5, HPS6* (HPAC BLOC-2 Deficiency); *HPS1, HPS4* (HPAC BLOC-3 Deficiency); *AP3B1, AP3D1* (HPAC AP-3 Deficiency). Carriers of pathogenic variants in genes associated with a HPAC BLOC-3 Deficiency exhibit a severe form of oculocutaneous albinism, progressive restrictive lung disease, and bleeding diathesis, whereas individuals with pathogenic variants in genes associated with a HPAC BLOC-2 Deficiency show a milder phenotype. Finally, carriers of pathogenic variants in AP3B1 or AP3D1 exhibit immunodeficiency.

The rare CHS presents with a variable degree of oculocutaneous albinism and a poor resistance to respiratory and cutaneous infections. The infections are generally fatal during infancy or in early childhood, but patients may also die of a chronic lymphohistiocytic infiltration known as the accelerated phase during the second or third decades of life. Unlike HPS, CHS does not seem to display locus heterogeneity and the only gene proved to cause CHS (*LYST*) is sited on chromosome 1q42.1–42.2; *LYST* codes for the lysosomal trafficking regulating protein CHS1 that is dysfunctional in CHS. The δ-SPDs mentioned are inherited in an autosomic recessive pattern, including thrombocytopenia with absent radius that may associate with a SPD. Instead, the deficiency of δ granules coincidental with Wiskott Aldrich syndrome is inherited in an X-linked recessive manner. The bleeding phenotypes of SPD are those expected from a platelet function defect and consist of easy and spontaneous bruising, mucocutaneous haemorrhages, haematuria and epistaxis. Patients with GPS, with δ-SPD or αδ-SPD usually have absent ADP and epinephrine-induced secondary aggregation waves, although the primary waves are present. Collagen-induced aggregation is absent or markedly reduced, whereas ristocetin-induced agglutination is normal.

Quebec platelet disorder is a rare autosomal dominant bleeding disease associated with abnormal proteolysis of α-granule proteins; platelets show increased stores of urokinase plasminogen activator and genetic studies show a tandem duplication of the urokinase plasminogen activator gene, PLAU. Intraplatelet generation of plasmin is thought to trigger degradation of stored α-granule proteins, increasing risks for a number of bleeding symptoms, including delayed-onset bleeding after haemostatic challenges that responds only to anti-fibrinolytic therapy.

Treatment

As a rule, there is no specific treatment for the vast majority of congenital platelet disorders. In congenital thrombocytopenias due to maternal use of drugs or chemical agents, thrombocytopenia recovers after few days to few weeks, as in the case of thiazide drugs. In surviving thrombocytopenic newborns infected with rubella or cytomegalovirus, platelet levels may return to normal after several months. In newborns with alloimmune congenital thrombocytopenia, platelets return to normal values in 14–21 days. Only severe cases need to be treated with washed platelets, corticosteroids or exchange transfusions. In general, principal treatments in patients with congenital platelet diseases are general measures aiming at avoiding bleeding and the use of supportive therapeutic approaches for controlling haemorrhages. However, since types and severity of bleeding vary in different patients, therapeutic approaches have to be individualised.

General measures

Patient education of patients is of great importance: patients and their parents have to be instructed to avoid trauma. Regular dental care may be helpful to prevent gingival bleeding. Drugs that impair platelet functions, such as acetylsalicylic acid-containing medications, should be avoided. In women, the use of oral contraceptives may be employed to prevent menorrhagia. Local measures, such as the application of firm pressure in the case of epistaxis, will usually suffice in the event of mild bleeding.

Drugs

Antifibrinolytic agents are useful as an adjunctive measure to prevent and control mild or moderate bleeding and may stop menorrhagia and other mild bleeding manifestations from mucous membranes, such as epistaxis: tranexamic acid, either orally at a dose of 15–25 mg/kg t.d.s. or intravenously at a dose of 10 mg/kg t.d.s. in the case of more serious bleeding. Antifibrinolytic agents represent the specific treatment for Quebec platelet syndrome.

Desmopressin (1-desamino-8-D-arginine vasopressin or DDAVP) is a mainstay of the therapy of patients with congenital platelet defects. Depending on platelet defect, administration of desmopressin may shorten the bleeding time. Intravenous, subcutaneous or intranasal administration of desmopressin increases factor VIII and VWF transiently by releasing them from storage sites and supporting platelet function. The drug is usually administered at a dosage of 0.3 μg/kg in 50 mL of saline by slow intravenous infusion (over 30 min to avoid possible hypotensive effects) or subcutaneously. In general, since the response to desmopressin varies amongst patients, but is constant in each patient, normalisation or improvement of the closure times of the PF100 or of bleeding time pre and post a test dose of desmopressin may identify those patients who may benefit from the drug. Recombinant activated factor VII (rFVIIa) has proven effective in the treatment of bleeding in haemophilic patients with inhibitors. The administration of rFVIIa in patients with congenital platelet disorders, such as BSS, SPD and Glanzmann's Thrombasthenia may stop spontaneous bleeding and/or may prevent bleeding during and after surgical procedures, though arterial and venous thromboembolism have been described after its use. Recombinant FVIIa is currently approved in Europe for prophylaxis and treatment of bleeding in patients with Glanzmann's thrombasthenia with antibodies to GP IIb-IIIa and/or HLA, and past or present refractoriness to platelet transfusion. The recommended dose is 90 μg/kg as a minimum of three intravenous boluses at 2-hour intervals.

With regards to the thrombopoietin agonists, a clinical trial has demonstrated the efficacy of eltrombopag in reducing the bleeding risk in the MYH9-related disease, in ANKRD26-related thrombocytopenia, in WAS, in the monoallelic BSS and in the ITGB3-related thrombocytopenia whereas the benefit of romiplostin in WAS derives from an observational study. Both agents provided pre-operative support in patients requiring surgery, minimising the use of platelet support hence of allo-immunisation.

Platelet transfusions

Platelet transfusions are employed to control severe haemorrhage in patients with thrombocytopenia or thrombocytopathies. Spontaneous as well as post-traumatic bleeding is common at a platelet count below 20×10^9/L and is extremely likely at a platelet count below 5×10^9/L. Extensive clinical experience has shown that control of bleeding is possible upon achievement of an adequate increase in the circulating platelet count. However, the coexistence of platelet dysfunctions has to be taken into account to correctly calculate the dosage of platelets required. Platelet transfusions are effective in controlling bleeding but may be responsible for transmission of infectious diseases, febrile reactions or development of alloimmunisation. Use of the thrombopoietic agents may substitute the need for peri-operative platelet support. In patients with thrombocytopathies, platelet transfusions should be employed only for the treatment of severe bleeding because of the risk of alloimmunisation.

The occurrence of platelet alloimmunisation is more frequent in patients lacking a membrane glycoprotein, such as in BSS and GT: indeed, the infusion of normal platelets into a donor with BBS or GT may stimulate the emergence of alloantibodies in the donor that may recognise the relevant platelet receptors in subsequent platelet transfusions causing platelet refractoriness. Similarly, a pregnant woman with BSS or GT may conceive a heterozygous fetus: If there is contamination of maternal blood with fetal blood, the mother could become sensitised to the relevant receptors and develop platelet refractoriness when given subsequent platelet transfusions.

Other measures

Splenectomy has generally no effect in congenital thrombocytopathies and in thrombocytopenias but has proven to be effective in some patients with inherited thrombocytopathies. Allogenic bone marrow transplantation may provide, in theory, an effective cure for inherited disorders involving platelet count or functions restoring a normal megakaryocytopoiesis. Indeed, it represents the only definitive therapy for patients with CAMT and remains the therapeutic modality of choice for classic WAS. Allogeneic bone marrow transplants have been successfully performed with complete correction in patients with amegakaryocytic thrombocytopenia with radioulnar synostosis, BSS and severe Glanzmann's thrombasthenia. However, the risks of an allogeneic bone marrow transplant are superior to those related to a bleeding tendency and, therefore, a transplant procedure is rarely required in patients suffering from congenital platelet disorders.

Conclusions

Over the recent years, a series of studies and reports have improved our understanding of the pathogenesis of congenital platelet disorders: new responsible genes have been identified and some patients have been characterised at the molecular level, allowing a deeper insight into the pathogenesis of inherited thrombocytopenias and thrombocytopathies (Table 44.5). Careful personal and family history taking, coupled with an

Table 44.5 Genes involved in congenital platelet disorders.

Disorder	Gene	Locus
Wiskott–Aldrich syndrome	*WAS*	Xp11.23-p11.22
X-linked thrombocytopenia	*WAS*	Xp11.23-p11.22
Congenital amegakaryocytic thrombocytopenia	*c-mlp*	1p34
Congenital amegakaryocytic thrombocytopenia and radioulnar synostosis	*HOXA-11*	7p15-p14.2
Schulman-Upshaw syndrome	*ADAMTS13*	9q34
May-Hegglin anomaly and Sebastian, Epstein, Fetchner syndromes	*MYH9*	22q11.2
Mediterranean macrothrombocytopenia	*GPIbα*	22q11.2, 17pter-p12
Jacobsen and Paris-Troussau syndromes	*FLI1*	11q23.3-11q24.2
Bernard-Soulier syndrome	*GPIbα*	22q11.2, 17pter-p12
	GPIbβ	22q11.2
	GPIX	3q21
Pseudo-vWD	*GPIbα*	22q11.2, 17pter-p12
ADP receptor defect	*P2Y12*	3q24-q25
	P2Y1	3q25
	P2X1	17p13.3
Epinephrine receptor defect	*α2AR*	10q24-q26
Collagen receptor defect	*α2*	5q23-q31
Glanzmann's thrombasthenia	*αIIb*	17q21.32
	β3	17q21.32
Hermansky-Pudlak	*HPS1*,	10q23.1-q23.2
	HPS2 (AP3B1), HPS3,	
	HPS4,	5q14.1
	HPS5,	
	HPS6,	3q24
	HPS7 (DTNBP1)	
	HPS8 (BLOC1S3)	22q11.2-q12.2
		11p15-p13
		10q24.32
		6p22.3
		19q13
Chediak-Higashi	*CHS1/LYST*	1q42.1-q42.2
Quebec platelet disorder	near *PLAU* locus	10q24
Thromboxane receptor defect	*TXBA2*	19p13.3

accurate physical examination are of major value for the evaluation of a patient presenting with bleeding due to congenital platelet disorders and are crucial in the selection of the appropriate laboratory tests (Figure 44.2). In most cases, this approach will allow the correct identification of the platelet defect. However, despite recent advances in knowledge, the molecular mechanisms underlying most of the congenital bleeding disorder remain unclear. The challenge to further our understanding of congenital platelet disorders must continue in order to design specific tools for their diagnosis, treatment and prevention. Gene therapy may represent a successful curative modality for congenital platelet disorders, whereas further trials with thrombopoietic agents will inform us about their efficacy and safety. Hopefully, refinements in such approaches will help provide targeted management for the majority of bleeders due to severe congenital platelet disorders.

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CHAPTER 45

Primary immune thrombocytopenia

45

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Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune bleeding disorder that affects both children and adults. Until recently, it was considered an autoantibody disorder in which platelets, opsonised with antiplatelet antibody, were removed prematurely by the reticuloendothelial system. More recent studies have shown that there is a relative platelet under-production in some patients, and there may be a role for CD8+ T cell mediated destruction of platelets and/or megakaryocytes.

Many patients have no clinical problems, but bleeding may occur. Other features of ITP are fatigue and an increased rate of thrombosis. ITP is unpredictable in its clinical course. Treatment is aimed at either reducing the platelet destruction through immunosuppression or increasing platelet production using thrombopoietin receptor agonists (TPORAs).

Clinical features

Paediatric and adult ITP have some differences in their underlying cause and presentation. In children, ITP often follows a viral illness or immunisation. The profound thrombocytopenia may be associated with extensive petechiae, purpura and bruises. There may also be bleeding from mucous membranes such as the nose or mouth. Despite the severity of the clinical features, many children do not need treatment and most will undergo spontaneous remission. Around 15% of children will develop chronic ITP, although this is higher in those presenting during adolescence, and may be more like 50%.

In adults, there is generally no prodromal illness and the patient may become aware of petechiae or excessive bruising and seek medical attention. ITP is often diagnosed by chance, for example during hospital admission for surgery or when a full blood count (FBC) is checked for other reasons.

ITP is heterogeneous. Many patients suffer few clinical problems related to their thrombocytopenia, while others have major bleeding from the outset. Although patients usually only bleed if the platelet count is less than 30×10^9/L, the platelet count alone is an unreliable predictor of outcome, and the clinical symptoms and signs of ITP are influenced by patient age, general health, comorbidities, medication and many other factors. In addition, there may rarely be an acquired platelet dysfunction caused by antibody binding to an important region of the glycoprotein molecules on the platelet surface. Autoantibodies reacting with glycoprotein (GP)IIb/IIIa may affect platelet aggregation, and anti-GPIb/IX autoantibodies can impair platelet adhesion to von Willebrand factor, causing unexpectedly severe bleeding for the level of platelet count. Other autoantibodies such as antiphospholipid antibodies, occur in up to 30% of patients with ITP, and these may affect platelet and vascular function, increasing rates of thrombosis. However, in general, unlike the thrombocytopenia that accompanies bone marrow failure, serious bleeding is not common in patients with ITP.

Reaching a consensus on terminology

Terminology for ITP was established by group of experts during a consensus meeting in order to help stratify disease and enable the comparison of different clinical trials. The key terminologies

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 45.1 Consensus Terminology definitions.

Phenomenon	Terminology and definition
Otherwise, idiopathic thrombocytopenia likely due to immunological mechanism	Immune thrombocytopenia
Type	Primary Secondary (SLE, HIV, hepatitis B, etc.)
Diagnostic criterion	Requires isolated platelet count $<100\times10^9$/L
'Acute' and 'chronic' categories	'Newly diagnosed' (0–3 months), 'Persistent' (3–12 months) 'Chronic' (>12 months)
Severe ITP	Where there is clinically relevant bleeding irrespective of platelet count

are listed in Table 45.1. With an increasing repertoire of new medications, refractory disease still needs to be better defined. Chronic ITP implies disease that has been present for 12 months or more from diagnosis.

Pathophysiology

ITP is thought to be a classic organ-specific autoimmune disease and, in common with other autoimmune diseases, there is a skewing of the Th1/Th2 response towards Th1 (proinflammatory). These are disorders in which there are antibodies or cells (B cells, T cells, antigen-presenting cells or others) that react against self-antigens. These may cause disease if the target tissue is damaged. The binding of antibodies to target cells results in clearance of the antigen from the body. The normal adaptive response, such as that seen in microbial infection, results in complete removal of the non-self-antigen. In autoimmune disease, however, there is continual production and incomplete clearance of the antigen, leading to perpetuation of the immune attack. In ITP the antigen is usually a platelet glycoprotein, found on megakaryocytes and platelets.

Antiplatelet antibodies and their targets

The autoantibodies involved in ITP are generally IgG, but IgA and IgM autoantibodies have also been reported. Opsonised platelets are removed prematurely by the reticuloendothelial system via an Fc-dependent mechanism. In addition, the autoantibodies may impair megakaryocyte growth and development, platelet release and may also induce apoptosis of megakaryocytes. The overall result of this is failure of platelet production.

There are various assays for measuring antiplatelet antibodies. Looking for the presence of platelet-associated IgG is of no value since this is found in non-immune as well as immune thrombocytopenia. More sophisticated assays, such as the monoclonal antibody-specific immobilisation of platelet antigens (MAIPA) assay, has greater specificity (90%) albeit with low sensitivity (50–65%). Using MAIPA, platelet-associated IgG and antigen capture assays, several platelet antigens have been characterised. These include GPIIb/IIIa (integrin αIIbβ3 and the fibrinogen receptor) and GPIb/IX (the von Willebrand factor receptor), which appear to be the most frequently involved. Less commonly, GPIa/IIa, GPIV and GPV are involved.

However, in many patients auto-antibodies are not detected, and responses to antibody depletion treatment are inconsistent suggesting there are other mechanisms of thrombocytopenia.

T cell involvement

The role of T cells in the development of ITP is becoming clearer. That T cells are involved has been known for some time, since the autoantibodies in ITP are predominantly of the IgG subclass, and isotype switching from IgM to IgG requires T-cell help. However, some 40% of patients with chronic ITP have no detectible autoantibodies yet are thrombocytopenic and appear to have true ITP. CD8+ T cells have been linked to the pathogenesis of many autoimmune diseases, such as type 1 diabetes. Studies by Wadenvik's group first showed that in some patients with ITP, T cells interact directly with platelets, inducing platelet lysis (Figure 45.1). We have recently confirmed this and shown that expanded CD8 T cell clones are present in patients with more refractory disease. These cells could also target megakaryocytes reducing platelet production. However, it is not yet clear how cell-mediated platelet destruction contributes to the severity of disease or to the difficulty of treatment in some patients with ITP.

Thrombopoietin levels in ITP

Thrombopoietin (TPO) is the principal growth factor involved in platelet production. Plasma TPO levels are elevated in patients with aplastic anaemia, chemotherapy-induced thrombocytopenia and other marrow failure syndromes. However, in ITP, levels of TPO are normal or only modestly elevated (Figure 45.2).

The liver produces TPO and this binds to TPO receptors on platelets and megakaryocytes. The free (unbound) TPO is able

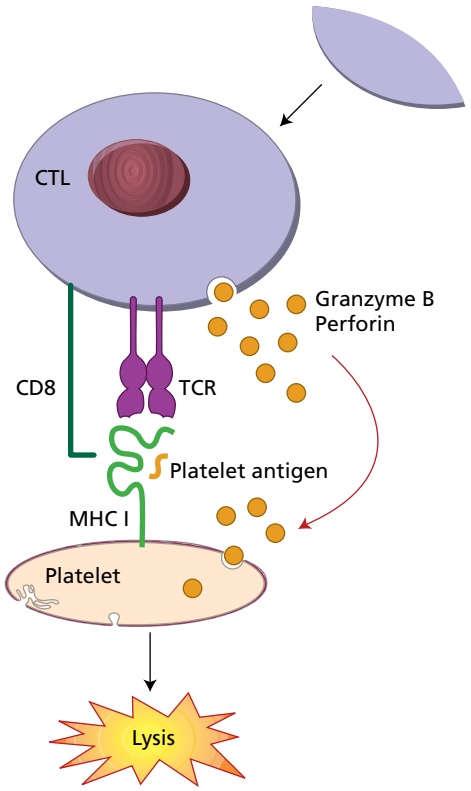

Figure 45.1 Model of cell-mediated cytotoxicity in chronic ITP. In the case of chronic ITP in the active phase, cytotoxic lymphocytes release toxic contents, such as granzyme B and perforin, and platelet lysis occurs.

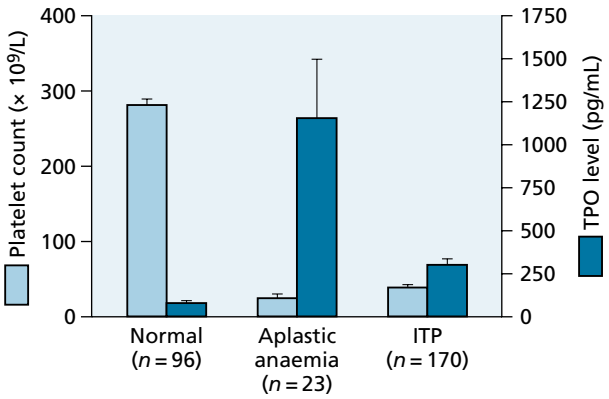

Figure 45.2 Thrombopoietin levels are usually normal or only slightly raised in patients with ITP, while in aplastic anaemia they are very raised, which can be an important test to distinguish between increased platelet destruction (such as ITP) or reduced platelet production (such as aplastic anaemia).

to stimulate the bone marrow to generate sufficient platelets to counterbalance the natural daily platelet losses through platelet senescence. If the megakaryocyte mass and platelet turnover are increased, such as in ITP, TPO is bound to the receptors on these cells, leaving little free TPO to stimulate undifferentiated bone marrow cells in patients with ITP. In addition, when older, desialylated platelets are removed in the liver, this triggers more TPO production. In ITP, there is a relative lack of TPO, which compounds the platelet losses that occur through increased destruction of platelets by the reticuloendothelial system.

Small studies also suggest that TPO levels can be used to predict response to thrombopoietin receptor agonists (TPORAs) but these assays are not widely available yet.

ITP is multifactorial

ITP has a multifactorial basis. Like other autoimmune diseases, loss of tolerance to a self-antigen alone is not sufficient to generate the autoimmune disorder. Patients probably require: (i) a specific set of genetic determinants, such as polymorphisms within major histocompatibility complex (*MHC*), *CTLA4* or other genes, (ii) dysregulation of the immune response (involving dendritic cells, T or B cells or all three) and (iii) an environmental 'trigger', which may be infectious, for example, a viral infection. Autoimmune disease arises only when all these determinants are present in an individual at the same time. This is further reinforced by the observation that self-reactive lymphocytes are commonly found in normal individuals.

In summary, ITP is much more complex than originally believed, with several mechanisms leading to thrombocytopenia (Table 45.2).

Possible triggers of ITP

The role of *Helicobacter pylori* in the development of ITP

This Gram-negative microaerophilic bacterium, the main cause of gastritis and peptic ulcer disease, has also been implicated in the development of gastric adenocarcinoma and mucosa-associated lymphoid tumours, and in some autoimmune disorders. Some studies have shown improvement in platelet counts in ITP patients positive for *H. pylori* following eradication of the bacterium. However, the data from different studies are

Table 45.2 Potential causes of thrombocytopenia in ITP.

- Autoantibody opsonisation of platelets leading to destruction by the reticuloendothelial system
- Autoantibody opsonisation of megakaryocytes, with inhibition of megakaryocyte growth, differentiation and platelet release
- Autoantibody-induced direct platelet or megakaryocyte apoptosis
- Relative thrombopoietin deficiency
- Molecular mimicry and immune complex formation
- T-cell mediated lysis of platelets

conflicting, with some centres showing a very high rate of response to eradication, while others have a low rate.

How *H. pylori* may initiate or perpetuate ITP is not known. Possibilities include molecular mimicry, where there is cross-reactivity between the antibody, the bacterium and platelet antigens.

COVID-19 and vaccine-induced ITP

During the pandemic, it became apparent that both SARS-CoV2 infection and the COVID-19 vaccines can precipitate ITP. Viral infection and the mRNA vaccines Pfizer-BioNTech usually cause transient ITP with spontaneous recovery. The ChAdOx1 nCoV-19 vaccine has been associated with more severe ITP, including haemorrhagic ITP although the number of cases per vaccine remain very rare. This is not unique to COVID-19 vaccines, as other vaccines and viral infections are also associated with ITP.

Natural history of ITP

The natural history of ITP is heterogeneous with some patients going into an early remission after steroid use, others responding to second-line therapy, and a smaller proportion developing refractory disease. There are no biomarkers to predict what type of ITP a patient will develop. While current treatments increase the platelet count, and improve the HRQoL, it is not known whether patients live longer on treatment or whether treatment alters the natural history of the disease. For many patients, ITP is a fairly minor disorder. Serious bleeding is not common and clinical sequelae of the disease are generally absent in patients with platelet counts above 30×10^9/L. There have been a few studies published looking at clinically relevant bleeding and platelet count. In normal individuals, there is a correlation between the platelet count and bleeding. The lower the platelet count, the more likely bleeding is to occur. In ITP most patients show little bleeding until the platelet count drops below 20×10^9/L. One study showed that no grade 4 bleeding occurred in patients with platelets greater than 10×10^9/L. A recent study of occult cerebral microbleeds was consistent with these findings, with patients only developing cerebral microbleeds if they had the lowest count less than 15×10^9/L.

ITP is not only a bleeding disorder. The Nordic registry has shown increased mortality in patients with ITP irrespective of bleeding. Patients with ITP also have an increased risk of developing thrombosis, and although uncommon this causes significant morbidity and mortality. Patient-reported outcome studies also report that fatigue and other HRQoL are impaired in ITP.

Diagnosis

Despite significant advances in our understanding of ITP, the diagnosis remains one of exclusion. A thorough history should be obtained, looking for diseases that might cause thrombocytopenia. The patient should be asked about bleeding during previous surgery or dentistry. The sites of bleeding should be determined.

A full physical examination should be normal, apart from the expected clinical signs associated with thrombocytopenia. In addition to the 'dry purpura' of ITP, there should be an examination of the mucous membranes, including the mouth and optic fundi looking for the presence of retinal haemorrhage. Enlargement of the liver, spleen or lymph nodes suggests an alternative diagnosis.

The laboratory investigation of ITP is straightforward. The FBC should confirm the isolated thrombocytopenia. There may be a degree of iron deficiency anaemia, but this should be in proportion to the clinical history. There should be no abnormal white blood cells or red cell fragments in ITP. If the latter are found, the underlying diagnosis may be thrombotic thrombocytopenic purpura, a disorder that is far more serious than ITP and which requires urgent treatment. Table 45.3 outlines the most useful tests in the diagnosis of ITP, along with other tests that are either of limited or no value.

Table 45.3 Investigation of suspected ITP.

Basic evaluation of ITP
- Patient history
- Family history
- Physical examination
- Full blood count
- Peripheral blood film
- Blood group (Rh) and reticulocyte count
- Direct antiglobulin test
- Quantitative immunoglobulin measurement
- Bone marrow examination*
- *Helicobacter pylori*
- HIV
- HCV

Tests that may be useful in selected cases
- Antiphospholipid antibody (including anticardiolipin and lupus anticoagulant)
- Antithyroid antibody and thyroid function
- Pregnancy test
- Antinuclear antibodies
- Viral PCR for parvovirus and cytomegalovirus
- Immunoglobulins and Lymphocyte

Tests of unproven or uncertain benefit
- Glycoprotein-specific antibody
- Thrombopoietin assay
- Reticulated platelet count
- Indirect platelet-associated IgG
- Platelet survival study
- Serum complement levels

* The need for bone marrow remains contentious and the indications for performing this test are discussed in Table 45.4.

Table 45.4 Indications for bone marrow examination in ITP.

- Failure to respond to, or relapse following, first-line therapy
- Presence of atypical clinical or laboratory features
- Before splenectomy (but see text)

Bone marrow examination

Previously, one of the mainstays of diagnosis was assessment of the bone marrow. The rationale behind this was the possibility of a patient having a marrow disorder such as leukaemia, lymphoma or infiltration. However, studies to date have shown that in patients with isolated thrombocytopenia, and with no atypical symptoms or signs, no cases of bone marrow pathology were detected. For this reason, recent guidelines do not recommend performing a bone marrow examination in all patients with ITP. If patients have unusual features such as multiple cytopenias, hepatomegaly, splenomegaly, lymphadenopathy, any clinical features suggesting the presence of a disease other than ITP, or they do not respond adequately to ITP treatments, then a bone marrow examination should be performed (Table 45.4).

Cytologically, the bone marrow in ITP generally shows normal development and maturation of all cell lines. Megakaryocyte numbers are typically normal or increased. During the clinical trials of the TPORAs, some patients developed increased reticulin fibrosis in the bone marrow. Subsequent reviews of patients with ITP who have not received any treatment have shown that bone marrow reticulin is increased in around two-thirds of patients. The increased reticulin seen in the TPORA studies is most likely due to stimulation of pre-existing reticulin.

Management

The two major international guidelines for the management of patients with ITP were updated in 2019. Both show that there are still limited data to help guide management.

In general, management is aimed to prevent major haemorrhage, the most feared event in patients with ITP. However, catastrophic bleeding is not common, and historically patients were over-treated, and the adverse effects of treatment, such as infection, were more common than bleeding symptoms. The main aim of treatment has therefore shifted away from trying to normalise the platelet count towards finding a strategy that will allow the patient to achieve a 'safe' platelet count. What constitutes a safe platelet count will vary from patient to patient, and also each patient with ITP will have differing requirements throughout their disease history. For example, a young patient with platelet count of 20×10^9/L should be safe to work and carry out normal day-to-day activities but would not be deemed safe if he or she wished to ski or undergo surgery. The safe platelet count would therefore be higher during these haemostatic challenges. The one-size-fits-all approach has failed to work over the years and has caused increased morbidity and mortality in patients due to the adverse effects associated with many of the treatments in current use.

There are many current treatments for ITP, although only a few have been approved (licensed) for this indication. Those that do have a license for ITP include corticosteroids, intravenous immunoglobulin (IVIg), intravenous anti-D, the thrombopoietin receptor agonists (TPORAs) eltrombopag, romiplostim and avatrombopag and the Spleen Tyrosine Kinase inhibitor (SYKi) fostamatinib. Rituximab also has considerable evidence in ITP but has not been submitted for licensing.

Initial treatment

First-line therapies comprise corticosteroids, IVIg and anti-D. These three treatments work fairly quickly (within 24–48 hours) and their efficacy rates are high at around 70–80% and are considered short-term treatment. The disadvantage of these and most of the other treatments is that the platelet count generally drops once the treatment is stopped. These treatments are seldom curative and must be considered palliative therapies. In addition, IVIg and anti-D are pooled blood products with their associated risks.

Second-line treatment

If patients fail to respond to first-line treatment, they may then be given a second-line drug, such as immunosuppression with azathioprine, mycophenolate mofetil or rituximab, or increasing the platelet count using a TPORA (Table 45.5).

Table 45.5 Treatment options after first-line therapy.

Non-approved second-line treatments
- Azathioprine
- Dexamethasone
- Methylprednisolone
- Ciclosporin
- Mycophenolate mofetil
- Cyclophosphamide
- Danazol
- Dapsone
- Vincristine
- Rituximab

Surgical second-line treatment
- Splenectomy

Approved second-line treatments
- Romiplostim
- Eltrombopag
- Avatrombopag
- Fostamatinib

Table 45.6 Target platelet counts for procedures.

Procedure	Target platelet count
Dentistry (non-invasive)	Any
Dentistry (fillings, local anaesthesia)	$\geq 30 \times 10^9$/L
Minor surgery	$\geq 50 \times 10^9$/L
Major surgery	$\geq 80 \times 10^9$/L

Short-term treatment

This refers to treatments lasting 2 weeks or more, which can be used when patients have a sudden drop in their platelet count leading to an unacceptably low platelet count, or when patients face haemostatic challenges such as planned surgery or dentistry, or other high-risk activity, where the main risk is excessive bleeding. Patients are generally started on treatment 1 or 2 weeks prior to the event, with regular monitoring of platelet counts. Table 45.6 shows the target platelet counts for the most common procedures.

Long-term treatment

This is required when patients have an unsupported platelet count that is too low for normal day-to-day activities. Generally, these patients will have failed other therapies or, if they responded to corticosteroids, they require unacceptably high doses to maintain a safe count. In this setting, the aim is not to achieve a specific count, but rather to move the platelet count into a 'safe' zone. This will vary from patient to patient. In general, once platelets exceed $15–20 \times 10^9$/L the risk of bleeding is low. Older individuals, or those with comorbidities or previous bleeding, will generally require a higher platelet count.

Many of the existing treatments used for long-term platelet control have known toxicities. The aim with long-term treatment is to use a treatment that has acceptable levels of toxicity, but which achieves the desired platelet count.

Thrombopoietin receptor agonists

Three TPORAs are now approved for ITP: romiplostim, a peptibody given as a weekly injection and eltrombopag and avatrombopag small molecules given as daily oral tablets. Romiplostim, eltrombopag and avatrombopag have undergone rigorous trials in adult patients with ITP both before and after splenectomy. Romiplostim and eltrombopag are also licensed for ITP in children – avatrombopag is currently in clinical trials, and met its primary end point in paediatric clinical trials. The study enrolled 75 subjects between 1 and <18 years. The primary endpoint of durable platelet response (6 out of 8 weekly platelet counts $\geq 50 \times 10^9$/L in the absence of rescue medication during weeks 5–12), was met in 28% of patients who received avatrombopag subjects in comparison with 0% of patients on the placebo arm ($p = 0.0077$, 95% CI 15.8–39.7). The efficacy rates for TPORAs are encouraging in all patients, and since they do not involve immunosuppression and are not corticosteroid-based, they appear to offer many advantages over other drugs.

From pivotal randomised clinical trials, responses to romiplostim were seen in 88% of non-splenectomised patients and in 79% of splenectomised patients. Concomitant ITP medication was reduced or stopped in 79% of subjects and there was a reduction in rescue medication. From clinical trials of eltrombopag, efficacy was reported at 79%, again with a reduction or cessation of concomitant medication in 59%, with reduction in bleeding reported at 76%. Both of these agents are now licensed for use as second-line treatment for ITP and are recommended second-line treatments in both International Consensus Guidelines and the American Society of Haematology Guidelines from 2019.

They are mostly very well tolerated. In some of the clinical trials, thrombotic events were reported in up to 6% of cases. Whether this is a treatment effect, or the increased risk of ITP is not clear. Initial concerns about the effect of long-term TPORAs on the bone marrow have not been realised.

In addition to the high efficacy, around 30% of patients treated with TPO-RAs may be weaned off the TPO-RA after 6 months if their platelet counts are stable and safe. This is termed 'treatment-free remission or TFR'. The mechanism for the TFR is not understood but there appears to be a reduction in inflammatory cytokines towards a more anti-inflammatory state.

Rituximab

This chimeric monoclonal antibody targets the CD20 antigen on B cells and is licensed for B-cell non-Hodgkin lymphoma and rheumatoid disease. Although it has not been submitted for licensing, there have been several studies of its use in ITP and other autoimmune haematological disorders including randomised controlled studies showing its efficacy in ITP. The largest review of rituximab in ITP was conducted by Arnold and colleagues, whose data suggest that around 60% of patients will respond to rituximab and approximately 40% of all patients will achieve a complete response. The responses are bimodal, with an early phase within 1–2 weeks and a second peak at about 6–8 weeks. Response duration varies from 2 months in partial responders to 5 years or more in about 15–20% of initially treated patients. A randomised controlled trial of rituximab versus placebo did not show any reduction in the rate of long-term treatment failure with rituximab, despite higher response rates and longer duration of response. A number of studies have suggested that women with a shorter duration of disease have better responses.

There are some safety concerns with rituximab and it should be used with caution in any patient who has positive hepatitis B core antibody status, since hepatitis B may be re-activated, and anti-viral prophylaxis is recommended for these patients. Adverse events associated with rituximab are usually mild or moderate, with a low incidence of infections. Severe infectious complications have been reported, although they are rare.

Fostamatinib: spleen tyrosine kinase inhibitor

Fostamatinib is the first spleen tyrosine kinase inhibitor to be licensed for use in ITP in the US in 2017 and in Europe in 2019 for the treatment of patients with ITP who have failed prior treatments. In the randomised controlled studies, the primary endpoint of stable platelet count $>50\times10^9$/L on 4 of 5 visits during weeks 14–24 was achieved in 18% of patients on fostamatinib and in 2% of patients on placebo; an overall response rate (a platelet count $\geq50\times10^9$/L on one or more visits in the first 3 months) was achieved in 43% of patients on fostamatinib and in 14% of patients on placebo. Platelet responses were long-lasting in the long-term extension study. Patients in these studies were mostly refractory to many other therapies. Ad-hoc analysis of the clinical trial showed higher response rates in patients who were within the first year of diagnosis. Interestingly there were no thrombotic events in the clinical trials. Adverse effects of treatment included gastrointestinal effects and hypertension.

Mycophenolate mofetil

Mycophenolate (MMF) is the most commonly used non-licensed immunosuppressive treatment in ITP. Most chronic studies show about 30% efficacy. A recent open-label randomised study showed lower relapse rates in patients who were randomised to receive MMF in addition to steroid therapy. Further studies are needed to compare immunosuppression with TPORAs as second-line therapy. MMF is contraindicated in pregnancy due to fetal toxicity.

Novel therapies in development

A number of classes of therapy are in late-stage clinical development. These target specific cells or molecules involved in ITP pathogenesis (see Figure 45.3). These agents include: anti neonatal Fc receptor drugs, Bruton tyrosine kinase inhibitors and anti-complement agents.

Anti-FcRn

The neonatal Fc receptor modulates the half-life of IgG and albumin by binding to IgG and releasing it from endothelial endosomes. As well as recycling normal IgG, the FcRn also recycles pathologic IgG. Blocking the FcRn causes IgG breakdown within the endosomes.

From recent clinical studies efgartigimod was well tolerated with a response rate of around 38%. Data thus far would appear to indicate that although the IgG levels fall this does not to increase the risk of infection.

Bruton tyrosine kinase inhibitors

Bruton tyrosine kinase, like syk, is involved in Fcγ signalling and is another potential target for ITP therapy. BTK is necessary for B cell development, function and antibody production. Several BTKIs have been developed for diseases such as chronic lymphocytic leukaemia (91). However, BTKIs have been reported to inhibit platelet aggregation which may result in bleeding, which is a concern in ITP. Although this was reported with ibrutinib it is not the case with rilzabrutinib.

Results from a recent Phase 2 study of rilzabrutinib in adult patients with relapsed or refractory ITP have been published. Fifty-nine adults, who had at least one prior response to treatment and a platelet count $\leq30\times10^9$/L, were given oral rilzabrutinib at 200 or 400 mg daily or 300 or 400 mg twice daily. The highest response rate was seen in the 400 mg twice daily group. Of 44 patients on this dose, 39% achieved the primary endpoint (two or more platelet counts $\geq50\times10^9$/L without the use of rescue therapy). Of the 33 patients who completed the study for more than 12 weeks, 17 of these (52%) responded. Of the responders half of these (50%) achieved a platelet count $\geq30\times10^9$/L by day 8. Rilzabrutinib was well tolerated with adverse events of Grade 1 or 2 only.

Anti-complement agents

Complement is believed to be involved in the pathogenesis of ITP for some patients. However, the contribution made by complement to ITP is not clearly understood. Early phase studies with sutimlimab, a monoclonal IgG C1s inhibitor, have been conducted. The patients treated were adults with ITP for >1 year and an inadequate response to ≥2 prior treatments. A total of 12 patients were treated of which 42% responded (platelet count $\geq50\times10^9$/L). Four patients (33%) achieved a platelet count of $\geq50\times10^9$/L for ≥70% of their study visits. One-third of patients responded in 2 days or less. No treatment-related adverse events were noted. Another complement inhibitor, iptacopan, a selective Factor B inhibitor, is currently undergoing Phase 2 clinical trials in ITP.

Other treatments in development

A number of other molecules are being developed. This includes other TPO receptor agonists lusutrombopag and heterombopag, as well as an anti-CD 38 molecule (daratumumab), and others. Anti-CD38 treatment may be useful for some with refractory ITP which may be caused by long-lived plasma cells. The proteasome inhibitor, bortezomib, has been used anecdotally in refractory ITP with variable results. However, this agent is not currently licensed for this indication.

Splenectomy

Splenectomy is a surgical option for patients who have relapsed after treatment with first- and second-line drugs. All guidelines recommend deferring splenectomy until at least 12 months from diagnosis. The procedure increases the lifespan of

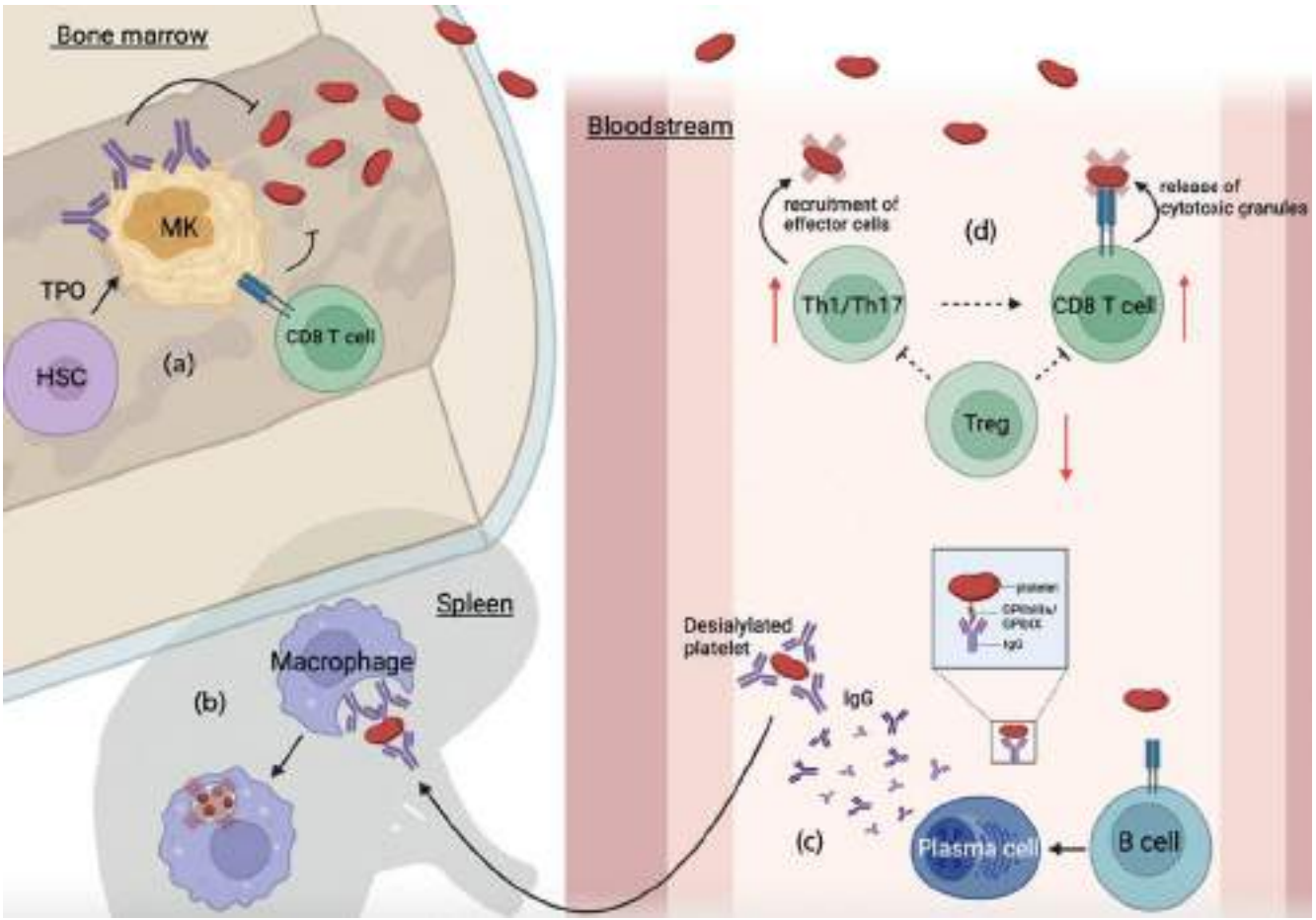

Figure 45.3 Pathogenesis of ITP. (a) Platelets are produced by megakaryocytes (MKs), which are derived from haematopoietic stem cells (HSCs) in the bone marrow. The process of thrombopoiesis is regulated by growth factors and cytokines, primarily thrombopoietin (TOP). (b) As platelets age, they become desialylated, marking them for clearance by the reticuloendothelial system, primarily in the spleen and liver. (c) In conditions like immune thrombocytopenia (ITP), platelets are destroyed due to autoantibodies that bind to abundant glycoproteins, particularly (GPIIb/IIIa and GPIb/IX) on both platelets and MK. The coating of autoantibodies on MKs interferes with their maturation, leading to impaired platelet release. Meanwhile, autoantibody-opsonised platelets are desialylated and subsequently phagocytosed by splenic macrophages via their Fcγ receptors (FCγRs). (d) In addition to autoantibody-mediated platelet destruction, abnormal T cell responses have been reported in patients with ITP. CD4+ T cell responses are skewed towards effector T helper (Th)1 and TH17 lineages and away from regulatory T cells (Tregs), leading to the breakdown of immune tolerance. Elevated levels of cytotoxic CD8+ T cells in ITP can directly induce platelet and MK apoptosis through T cell receptor (TCR)-mediated release of cytotoxic granules. (*Source:* Created with BioRender.com).

antibody-coated platelets and may reduce antibody production. Responses are seen in about two-thirds of patients, who achieve a normal platelet count, and the response is often sustained with no additional therapy for at least 5 years. Even patients who do not have a complete response may still expect a partial or transient increase in platelet count. Around 14% of patients do not respond and approximately 20% of responders will relapse months or years later.

Splenectomy has a low but definite peri- and post-operative morbidity and mortality but the rates have been much reduced by the use of laparoscopic splenectomy.

Predicting the response to splenectomy

Clinicians have long sought possible predictors of response to splenectomy. Potential predictors have included response to oral corticosteroids, IVIg and others. Indium-labelled autologous

platelet scanning appears to be the most sensitive predictor of response to splenectomy. However, indium scanning is not available in every hospital and is limited to only a few centres worldwide.

Prevention of infection after splenectomy

Splenectomised patients are at long-term risk for opportunistic postsplenectomy infection (OPSI) with encapsulated bacteria (see also Chapter 5). OPSI is often rapidly progressive and has a poor outcome. The main organisms responsible for sepsis in splenectomised patients are pneumococci (50%), meningococci and *Haemophilus influenzae*.

In order to prevent OPSI, patients should be given prophylactic polyvalent pneumococcal vaccine at least 4 weeks prior to, or 2 weeks after, splenectomy, with re-vaccination according to local guidelines. *H. influenzae* b and meningococcal C conjugate vaccinations should also be administered prior to splenectomy. Re-vaccination in susceptible individuals for *H. influenzae* should be administered according to local guidelines.

In the UK, the Chief Medical Officer advises the long-term use of oral antibiotics in the form of phenoxymethylpenicillin 250 mg twice daily, or equivalent, or erythromycin 250 mg twice daily if the patient is allergic to penicillin. However, the need for lifelong antibiotic prophylaxis remains unproven.

Patients with refractory ITP

The Terminology Consensus document published in 2009 defines refractory ITP as that where the patient has undergone splenectomy which has failed or relapsed, and who has 'severe' ITP (clinically relevant bleeding) or a risk of bleeding. Some 20% or more of adult patients fall into this category. Clinically, patients with refractory ITP pose a major challenge since, by definition, they are resistant to many of the treatments in current use. They often have low platelet counts, in addition to bleeding that is difficult to control since the disease is unresponsive to conventional therapies.

Possible options for patients with refractory ITP are combinations of treatment including TPORAs and immunosuppression and combined chemotherapy – although this is often poorly tolerated. Many patients may not need treatment, even with low platelet counts.

ITP in children

Children with ITP are treated based on clinical bleeding, quality of life and not just platelet count. Severe bleeding is uncommon in children with ITP, even when the platelet count is very low ($<10\times10^9$/L). The incidence of intracranial haemorrhage (ICH) in children with ITP is around 0.1–0.5%. As with adult ITP, there is no method to predict which children will develop ICH. In general, however, ICH is commoner in patients with refractory or resistant disease than at initial presentation. Investigation of childhood ITP is similar to that in adults, although the incidence of *H. pylori* infection is much lower and there is no need to screen for this.

'Watch and wait' policy

Around 70% of children with acute ITP do not have significant bleeding symptoms and can be managed without drug therapy. Admission to hospital should be reserved for children with clinically significant bleeding, such as severe epistaxis or gastrointestinal or other bleeding. Most children with minor, mild or moderate symptoms can be safely managed in the outpatient setting. The emphasis is towards treating symptoms rather than platelet counts, since the platelet count is a surrogate marker and does not accurately predict clinical outcome.

General measures for persistent and chronic ITP in children

Many children stabilise with an adequate platelet count and have no symptoms unless injured. In children who are less than 10 years of age at diagnosis, spontaneous remission is very likely to occur, and expectant management can continue depending on the risk of bleeding and the degree of activity restriction of the child. The onset of menstruation may be problematic and can be managed with antifibrinolytic agents and the combined contraceptive pill.

Management of chronic ITP in childhood should be tailored to each individual child and situation, and therapy should be effective and it should not carry more risk than the untreated condition.

Treatment options in childhood ITP

Children with severe bleeding symptoms should be treated. Treatment should also be considered in children with moderate bleeding or those at increased risk of bleeding and for patients whose lifestyle is restricted due to ITP.

First-line treatment in children

If treatment is deemed to be required, options include IVIg, IV anti-D and corticosteroids.

IVIg raises the platelet count in more than 80% of children and does so more rapidly than steroids or no therapy. Transient side-effects are seen in 75% of children. Intravenous anti-D immunoglobulin can be given to Rh(D)-positive children as a short infusion and is useful in the outpatient setting. Mild extravascular haemolysis is common, with a mean drop in haemoglobin of 10 g/L. Like IVIg, anti-D is a pooled blood product, but it is derived from a smaller donor pool. The safety profile of anti-D is good with no reported transmission of infection to date. However, there have been reports of severe intravascular haemolysis leading to disseminated intravascular coagulation, most of which have been fatal. Prednisolone at a dose of 1–2 mg/kg daily for a maximum of

14 days is effective in around 75% of children (platelet count $>50\times10^9$/L) within 72 hours. More prolonged high-dose corticosteroid regimens are associated with increased toxicity.

Second-line treatment for paediatric ITP

Thrombopoietin receptor agonists (TPORAs) are the recommended next line of treatment for children who have significant bleeding, or who develop chronic ITP. Immunosuppressive treatments including rituximab and MMF can be considered in those who are refractory to TPORAs and continue to have symptoms.

ITP in pregnancy

Thrombocytopenia occurs in 5% of pregnancies, but most of these cases are gestational rather than immune-mediated (see also Chapter 53). The diagnosis of ITP involves the exclusion of other causes of thrombocytopenia during pregnancy. Patient history, physical examination, blood count and blood film examination are used in non-pregnant patients. The work-up of a pregnant patient with ITP is essentially the same as that of a non-pregnant patient. There are some conditions specific to pregnancy and these should be considered when investigating a pregnant patient with thrombocytopenia (Table 45.7).

Laboratory investigation of ITP in pregnancy

ITP in pregnancy, as with other types of ITP, is a diagnosis of exclusion. All investigations carried out in pregnancy are aimed at excluding conditions that may result in thrombocytopenia. Some disorders such as thrombotic thrombocytopenic purpura and HELLP syndromes require urgent diagnosis and treatment since the mortality rates are high (Table 45.8).

Management of ITP in pregnancy

There should be close collaboration between the obstetrician, haematologist, obstetric anaesthetist and neonatologist. Treatment is largely based on the risk of maternal haemorrhage. Throughout the first two trimesters, treatment is initiated when the patient is symptomatic and/or when platelet counts fall below 20×10^9/L, or when it is necessary to produce an increase in platelet count to a level considered safe for procedures such as obstetric delivery or epidural anaesthesia. Patients with platelet counts of $20–30\times10^9$/L or more do not require routine treatment.

Table 45.7 Causes of maternal thrombocytopenia in pregnancy.

- Gestational thrombocytopenia
- Pre-eclampsia, HELLP syndrome and disseminated intravascular coagulation
- Folate deficiency
- Massive obstetric haemorrhage
- Acute fatty liver

HELLP, haemolysis, elevated liver enzymes and low platelets.

Table 45.8 Investigation of suspected ITP in pregnancy.

- Coagulation screening (prothrombin time, PT)
- Activated partial thromboplastin time (APTT)
- Fibrinogen assay
- Liver function tests including bilirubin, albumin, total protein, transferases, γ-glutamyl transferase and alkaline phosphatase
- Antiphospholipid antibodies, including anticardiolipin antibodies and lupus anticoagulant
- SLE serology
- Review of the peripheral blood film
- Reticulocyte count
- Bone marrow examination: not required to make the diagnosis of ITP in pregnancy

Delivery

A platelet count above 50×10^9/L is generally acceptable for standard vaginal or caesarean delivery. For epidural anaesthesia, a platelet count of 75×10^9/L is required, although this is not evidence-based.

Treatment options in pregnancy

First-line treatment options for ITP in pregnancy are similar to those of other adult ITP patients, namely corticosteroids and IVIg. There is limited evidence for the use of intravenous anti-D, splenectomy, azathioprine, rituximab and TPORAs.

Other agents, such as mycophenolate should not be used in pregnancy because of known teratogenicity.

Corticosteroids

These are the most cost-effective option. Prednisolone is initially given at a dose of 10–20 mg/day and may then be adjusted to the minimum dose that produces a haemostatically effective platelet count. At least 90% of the administered dose of prednisolone is metabolised in the placenta by 11β-hydroxlase, but high doses may have an effect on the fetus.

Intravenous immunoglobulin

If prolonged high-dose steroid therapy is required or significant side-effects occur, or a rapid platelet increase is required, IVIg should be considered. The conventional doses of IVIg and likely response rates are similar to those seen in non-pregnant patients.

Options for maternal ITP refractory to first-line treatment

As with non-pregnant adults, combining first-line treatments in the refractory patient may be appropriate in the weeks prior to delivery. High-dose methylprednisolone (1000 mg), possibly in

combination with IVIg or azathioprine, has been suggested as a treatment for pregnant patients' refractory to oral corticosteroids or IVIg. Azathioprine has been used for many years in the post-transplant setting and this drug appears to be safe in pregnancy, though the response is generally slow. Splenectomy in pregnancy is rarely required, but if essential is best carried out in the second trimester.

Rituximab has been used during pregnancy in patients with ITP and with other autoimmune disorders and appears safe when used in the second trimester. Both azathioprine and rituximab can help to reduce the amount of steroids used.

Increasing case reports have shown safety and efficacy of TPORAs in the third trimester in patients with very refractory disease but should be used with caution.

Management of the neonate (of mothers with ITP)

Thrombocytopenia in the neonate (from mothers with ITP) accounts for 3% of all cases of thrombocytopenia at delivery. The fetal or neonatal platelet count cannot be reliably predicted from the maternal platelet count. After delivery, a cord blood platelet count should be determined in all cases. Intramuscular injections (such as vitamin K) in the fetus should be avoided until the platelet count is known. Those infants with subnormal counts should be observed clinically and haematologically, as the platelet count tends to fall further to a nadir between days 2 and 5 after birth.

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CHAPTER 46

Thrombotic thrombocytopenic purpura (congenital and acquired) and haemolytic–uraemic syndrome

46

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Thrombotic thrombocytopenic purpura (TTP) was first described in 1924 by Moschcowitz in a 16-year-old girl who died after an acute illness presenting with a pentad of signs and symptoms (anaemia, thrombocytopenia, fever, hemiparesis and haematuria). At postmortem examination disseminated thrombi, principally made of platelets, were found in the terminal circulation of multiple organs. Over the next three decades, other cases were described, more frequently but not exclusively in women, occurring alone (idiopathic) or in association with other diseases of autoimmune, infectious or neoplastic nature or with drug intake and during pregnancy. It was also understood that thrombocytopenia was caused by platelet consumption due to the widespread deposition of thrombi in the microcirculation and that anaemia was due to intravascular haemolysis following the mechanical fragmentation of red cells (schistocytes) in blood forced to flow through partially occluded vessels.

Thirty-one years after Moschowitz, Gasser described a syndrome called haemolytic–uraemic syndrome (HUS). In common with TTP, HUS was characterised by mechanical haemolytic anaemia, consumptive thrombocytopenia and microvascular thrombosis, but differed because there were minimal neurological symptoms but severe renal damage with uraemia, often heralded by episodes of bacterial haemorrhagic enterocolitis. Subsequently, it became apparent that a clear distinction between the two syndromes was difficult. Although thrombocytopenia, anaemia and multiorgan ischaemic symptoms due to disseminated thrombus formation in the terminal circulation were common features, the prevalence of neurological over renal symptoms could not clearly differentiate TTP from HUS and vice versa. The term thrombotic microangiopathy (TMA) was then proposed, meant to emphasise the common pathology of the two syndromes with no implication of the prevalence of neurological or renal symptoms.

Even though it had been postulated that in TTP disseminated thrombus formation in the microcirculation was due to the presence of substance(s) that aggregated platelets intravascularly, the putative aggregating agent remained elusive until 1982, when Moake and colleagues demonstrated that the plasma from 4 patients with TTP contained ultralarge, highly thrombogenic forms of the multimeric glycoprotein von Willebrand factor (VWF), a major adhesive moiety contained in endothelial cells, platelets and plasma. VWF multimers of particularly high molecular weight (ultralarge) bind avidly to the platelet receptor glycoprotein (GP)Ib-IX-V and aggregate platelets in conditions of high shear stress such as those occurring in the microvascular beds. Accordingly, ultra-large VWF was postulated to be the aggregating agent involved in the formation of thrombi in the microcirculation of TTP patients.

It remained to be explained why normally absent ultra-large multimers were present in the plasma of TTP patients. Even though the deficiency or dysfunction of one or more enzymes physiologically disposing of them had been postulated, it was

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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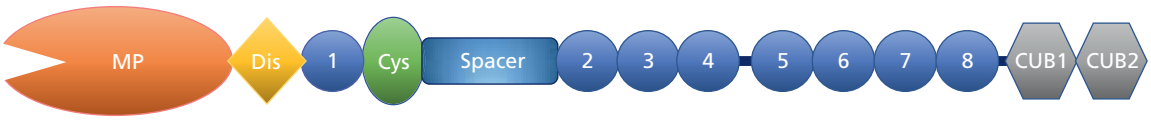
Companion website: www.wiley.com/go/mead/HPH8e



Mp = Metalloprotease
Dis = Disintegrin
Cys = Cysteine rich domain
1–8 = Thrombospondin like domains
CUB = *C*omplement components C1r/C1s, *U*rinary epidermal growth factor, *B*one morphogenetic protein-1

Figure 46.1 Domain structure of ADAMTS13. TSP1: thrombospondin 1; RGDS: Arg-Gly-Asp-Ser; CUB: *C*omplement components C1r/C1s, *U*rinary epidermal growth factor, *B*one morphogenetic protein-1.

not until the late 1990s that the research groups led by Furlan and Tsai independently showed that the link between ultra-large VWF multimers and TTP was a metal ion-dependent plasma metalloproteinase of 190 kDa, identified in 2001 by Zheng and colleagues as a new member (the 13th) of the ADAMTS (*A* *D*isintegrin *A*nd *M*etalloprotease with *T*hrombo*S*pondin type-1 repeats) family of metalloproteinases (Figure 46.1).

The physiological function of this protease, contained in plasma and platelets, is to reduce the size of VWF by cleaving ultra-large multimers as soon as they are secreted from endothelial cells (Figure 46.2), thereby avoiding their presence in plasma leading to *in vivo* platelet aggregation and heightened thrombus formation. Most importantly, both the investigators made the seminal and still cogent observation that ADAMTS13 was deficient in the plasma of TTP patients but not in those with HUS. This observation challenged the unitarian theory of TTP and HUS as different clinical manifestations of the same disease mechanism (TMA) and generated the current views that TTP is due to very low plasma levels of the VWF-cleaving protease whereas HUS, characterised by moderately reduced to normal plasma levels of the protease, is often mechanistically related to alterations of components of the complement system. Thus, TTP and HUS are different diseases, even though there is some degree of overlap of signs and symptoms owing to the common pathology of disseminated thrombotic microangiopathy. Accordingly, an accurate differential diagnosis is warranted, because different first-line therapies are currently employed for these life-threatening diseases (plasma exchange and the VWF inhibitor caplacizumab for TTP and the complement inhibitor eculizumab for the atypical, complement-mediated forms of HUS).

Thrombotic thrombocytopenic purpura

TTP is a rare disease with an estimated yearly incidence of approximately five cases per million, even though greater awareness and improved diagnostic facilities give the impression that incidence is currently increasing. *Congenital TTP*, also known as Upshaw–Schulman syndrome, caused by mutations in the gene encoding ADAMTS13 and transmitted as an autosomal recessive trait, is often but not exclusively clinically manifested at birth or during childhood. *Acquired TTP* (preferably identified as immune-mediated TTP) is due to the development of autoantibodies that inactivate or bind ADAMTS13. Mortality of both TTP types was very high (80–90%) until plasma exchange

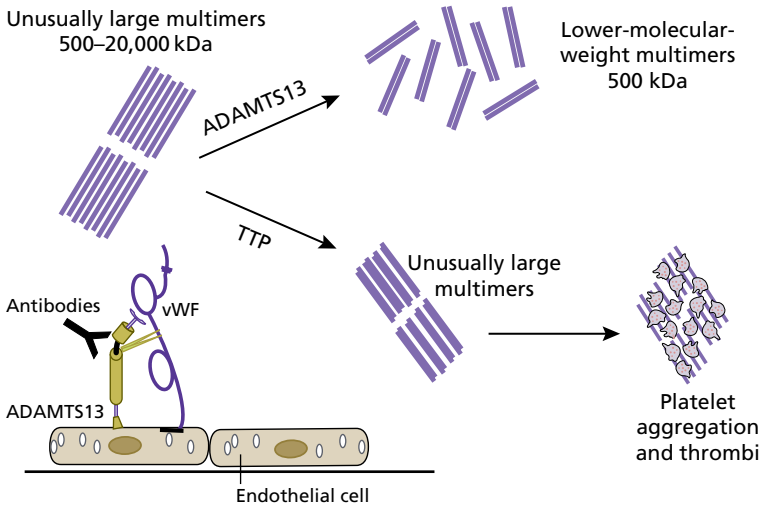


Figure 46.2 Pathophysiology of TTP. VWF is secreted from endothelial cells in the form of ultra-large multimers that anchor to endothelial cell surfaces but are also released into the circulation. ADAMTS13 cleaves a Tyr–Met bond in the A2 domain of VWF, thus severing the ultra-large multimers. The inherited or acquired deficiency of ADAMTS13 leads to failure of VWF cleavage and thus to the persistence in plasma and onto endothelial cells of ultra-large multimers, which aggregate platelets, especially in conditions of high shear forces.

(PEX) was introduced for treatment but is still high (10–20%), despite the dramatic improvements recorded after the adoption of this procedure. Because of the wide spectrum of presenting symptoms and associated comorbid conditions, cases of TTP may be initially seen by a variety of physicians other than haematologists – such as neurologists, nephrologists, oncologists and gynaecologists – and this sometimes hinders the prompt recognition of the disease.

Pathology and mechanism of TTP

The pathological basis of both congenital and acquired TTP is the disseminated formation in the microcirculation of platelet thrombi, associated with relatively little endothelial cell injury and fibrin content but abundant intrathrombus VWF. Microthrombi are found in multiple organs (mainly brain, kidney, in myocardium causing sudden death, adrenal gland, digestive tract and pancreas) and lead to multiorgan failure, whereas thrombi in large arterial and venous blood vessels are usually lacking. The current mechanistic model implies that endothelial cells, perturbed in the context of varied triggering conditions (Table 46.1), secrete greater amounts of ultra-large VWF multimers that, in situations of congenital or acquired ADAMTS13 deficiency, remain uncleaved and aggregate platelets in conditions of high fluid shear stress, resulting in massive microvascular thrombosis, ischaemic organ damage, consumptive thrombocytopenia and mechanical haemolytic anaemia (Figure 46.2).

Congenital TTP

Congenital TTP is very rare and represents no more than 4% of all TTP cases with an annual incidence of less than 1 per million. It more often occurs early after birth or during infancy, although the disease may become clinically manifest in adulthood at the time of infections or pregnancy. Congenital TTP is caused by homozygous or more frequently double heterozygous biallelic mutations in the *ADAMTS13* gene (located on chromosome 9q34) that affect protein secretion or function. According to the International Hereditary TTP Registry, more than 200 mutations have been identified to date. Their location on *ADAMTS13* reveals no common hotspots, even though 75% of the missense mutations are in the gene portion that encodes the N-terminal domain of the molecule. The majority of mutations are missense and the remaining are nonsense, splice-site and frameshift variants that lead to the formation of truncated forms of the protease. Of the mutations characterised by means of *in vitro* expression studies, the majority affect ADAMTS13 biosynthesis, intracellular trafficking and secretion and/or proteolytic activity.

Notwithstanding the consistent hallmark of severe ADAMTS13 deficiency unaccompanied by inhibitory antibodies, clinical presentation is variable, with between-patient differences in terms of age of onset, severity, number of relapses and need for long-term prophylactic replacement therapy (see more below). The type of gene mutation may account for some of this heterogeneity and influence phenotype severity: mutations in the portion of the gene encoding the N-terminal domains of the protease may be associated with more severe disease in terms of earlier age at first episode and higher rate of recurrence. Pertaining to the potential triggers of acute episodes, conditions such as pregnancy and infections are believed to act by inducing an increase in VWF levels exceeding the levels that tiny residual amounts of protease activity are normally able to dispose, leading to the precipitation of acute TTP episodes.

Table 46.1 Underlying conditions and diseases associated with immune-mediated TTP.

- Pregnancy and postpartum
- Infections (particularly HIV)
- Drugs (quinine and quinidine, clopidogrel, cyclosporin, interferon-α, statins)
- Chemotherapy (mitomycin, cisplatin, gemcitabine)
- Organ transplantation
- Autoimmune disease (lupus erythematosus, rheumatoid arthritis, scleroderma)
- General and cardiac surgery

Acquired TTP

Immune-mediated TTP accounts for the great majority of clinical cases and occurs mainly in adults, often as a primary disease in the absence of any obvious trigger or comorbidity. However, it can also arise in association with such conditions as immune-mediated diseases, infections, drug intake, pregnancy, sepsis, tumours and organ transplantation (Table 46.1). Acquired TTP is due to the development of autoantibodies that inhibit the enzymatic activity of ADAMTS13 and/or bind the protease, thus leading to its accelerated plasma clearance through opsonisation and/or other mechanisms. Anti-ADAMTS13 autoantibodies are usually of the IgG class, although in a few cases, they are of the IgA and/or IgM isotype. Epitope mapping of anti-ADAMTS13 showed that in the majority of cases they are polyclonal antibodies of the IgG4 subclass, with a primary epitope in the Cys-rich spacer domain of the protease that is essential for efficient VWF-cleavage activity and crucial for the interaction between the protease and its substrate. However, cases of autoimmune TTP are also observed in patients with IgG molecules that react with the TSP1 2–8 and CUB domains of ADAMTS13 (Figure 46.1). Ultra-large VWF multimers are not constantly detected in patient plasma, perhaps because of an imbalance between their release in plasma from endothelial cells and heightened binding to platelets, so that larger multimers may be even lacking in plasma.

Clinical and laboratory findings in TTP

The presence of both consumptive thrombocytopenia and mechanical haemolytic anaemia is essential for a clinical diagnosis of both congenital and acquired TTP. Platelet count is very low in the acute phase, with values of 30×10^9/L or less. Signs of mechanical haemolytic anaemia (haematocrit usually <20%) include the presence of schistocytes on peripheral blood smears, reticulocytosis (>2.5%), high indirect bilirubin (>2mg/dL), very low or unmeasurable haptoglobin and negative direct Coombs test. High serum lactate dehydrogenase (LDH), usually in excess of 1000 U/L, is a sensitive albeit non-specific sign of red cell destruction and tissue necrosis due to microthrombotic organ ischaemia and is useful to monitor disease evolution. The main presenting clinical features and signs in acute TTP are in Table 46.2. Neurological symptoms (coma, stroke, seizures or focal signs such as motor deficits and aphasia) typically fluctuate in presentation and severity as a result of the ongoing formation/clearance of thrombi in the cerebral microcirculation. Other neurological symptoms (such as headache, blurred vision, ataxia or mental status changes) are less typical. Chest and abdominal pain are also frequent. However, in the majority of cases disease onset is characterised by non-specific signs and symptoms such as fatigue, nausea and fever. Even though the majority of patients with TTP have serum creatinine levels no higher than 2.0 mg/dL, signs of renal involvement such as microscopic haematuria and mild proteinuria are frequent. There is little or no alteration of the coagulation and fibrinolysis systems, even though D-dimer plasma levels may be moderately increased.

ADAMTS13 laboratory testing

Measuring ADAMTS13 in plasma is currently possible owing to the availability of laboratory assays of enzyme activity. These were initially based on the degree of degradation of plasma-derived or recombinant VWF by ADAMTS13 contained in test plasma, but now the most widely used assays are based upon synthetic VWF peptides containing the protease cleavage site. All available assays measure ADAMTS13 activity in static conditions and thus fail to reflect the *in vivo* physiological blood flow conditions necessary for optimal protease activity.

- The current use of peptides as FRET (fluorescence resonance energy transfer) substrates instead of full-length VWF in enzyme immunoassays may fail to detect a few ADAMTS13 deficiencies, since these assays measure the metalloprotease activity independently of the exosite interactions with other VWF domains. Similar problems may occur when ADAMTS13 autoantibodies develop against these exosites.
- Activity assays are often available only in specialised laboratories and usually not in an emergency time frame.
- Immunoenzymatic assays which measure plasma ADAMTS13 antigen usually give low levels, but are of limited clinical value, because they measure only the immunoreactive protein and not the protease functional activity.
- With regard to the laboratory detection of autoantibodies, most of them inhibit the enzymatic activity of ADAMTS13, so that they can be titrated *in vitro* by classical mixing studies, using dilutions of mixtures of serially diluted plasma from patients and normal individuals. Direct antibody Western blotting and ELISA are also available.

Clinical applications of ADAMTS13 assays

Unmeasurable or very low plasma levels of enzymatic activity (<10%) are required to unequivocally establish a diagnosis of inherited or acquired TTP (Table 46.3). The detection of anti-ADAMTS13 helps to distinguish congenital from acquired TTP and offers a guide to disease prognosis and treatment of the immune-mediated forms with immunosuppressive drugs. After remission from an acute TTP episode approximately one-third or more patients develop chronic recurrent disease, particularly those who present with unmeasurable ADAMTS13 activity (Table 46.3) and still detectable anti-ADAMTS13 autoantibodies during first remission.

Table 46.2 Presenting clinical symptoms and signs in acute TTP.

Thrombocytopenia	Epistaxis, bruising, petecchiae, microscopic haematuria
Neurological symptoms – often fluctuating	Confusion, headache, paresis, seizures and aphasia, dysarthria, visual problems, fatigue
Fever (>37.5 °C)	Not constantly present
Jaundice	Resulting from mechanical haemolytic anaemia
Renal	Proteinuria, microhaematuria
Cardiac	Chest pain, heart failure, hypotension
Gastro-intestinal tract	Abdominal pain, nausea

Table 46.3 Clinical applications of *ADAMTS13* testing in acute TTP and aHUS.

Stage	ADAMTS13 deficiency (<10%)	Diagnostic implications
Acute phase	Yes	TTP is confirmed
	No	TTP is excluded, suspect aHUS or other TMAs
Remission	Yes	Risk of relapse

Recently the International Society on Thrombosis and Haemostasis (ISTH) developed TTP guidelines which apply rigorous methodologic standards to the diagnosis and management of adult patients with a suspected TTP. In particular, the ISTH guidelines on TTP diagnosis pointed out the importance of ADAMTS13 testing, emphasising that the use of the clinical scores (such as the French and PLASMIC scores) supports clinical judgement in differential diagnosis and decision for treatment but cannot replace *ADAMTS13* testing. The ISTH guidelines also emphasise the importance of providing clinicians with a timely access to measurement of plasma ADAMTS13 activity (and inhibitor or anti-ADAMTS13 IgG) for the optimal management of newly diagnosed patients with TTP. They also concluded that the management of TTP relapses in a previously diagnosed patient could be safely undertaken on clinical grounds with no need for a confirmatory *ADAMTS13* test.

Differential diagnosis with HUS

Typical HUS is usually easily distinguishable from TTP owing to the occurrence of diarrhoeal prodromes and the presence of more frequent and severe symptoms of renal impairment. It is sometimes more difficult to distinguish TTP from atypical HUS, notwithstanding the higher prevalence of neurological symptoms in the former and more severe renal failure in the latter (see more below). However, reliance on prevalent organ symptoms is misleading, because approximately half of cases with atypical HUS have neurological symptoms and a few of them may present, at least initially, with normal creatinine. Other issues of differential diagnosis materialise with conditions that present themselves as TMAs, all being characterised by consumptive thrombocytopenia and mechanical haemolytic anaemia (Table 46.4)

A severe deficiency of ADAMTS13 unequivocally distinguishes TTP from HUS and other TMAs (Table 46.3). However, because results of *ADAMTS13* testing often have a turnaround time incompatible with the early application of a life-saving therapy such as PEX, efforts were made to identify simple laboratory data that predict before PEX which subjects are more likely to be severely deficient in ADAMTS13 activity and thus have TTP. Pre-treatment platelet count lower than 30×10^9/L and creatinine less than 2.3 mg/dL predict severely deficient ADAMTS13 activity (<10%) with a sensitivity of 98%, specificity 48%, positive predictive value 85%, negative predictive value 93%. These criteria led to the development of the so-called French Score, widely used in clinical practice for its simplicity and clinical value to proceed to implement PEX. More recently, the PLASMIC score added to these simple biomarkers other positive or negative data recorded at presentation, such as the degree of haemolysis epitomised by unmeasurable haptoglobin, a mean corpuscular volume lower than 90 fL, a prothrombin time INR <1.6, no active cancer or history of organ transplantation. The ensuing scores are used to define cases at higher, intermediate or low risk of being TTP (Table 46.5). A PLASMIC score of 5 or more has a diagnostic sensitivity of 99% and specificity of 57%. On the other hand, moderate or mild thrombocytopenia and severe renal insufficiency almost eliminate the likelihood of severe ADAMTS13 deficiency, indicate that the diagnosis is not TTP and lead to suspicion of HUS in patients presenting with TMA. However, as emphasised in the ISTH guidelines, the final confirmation of TTP diagnosis requires *ADAMTS13* testing. Even though a dysregulation of the complement system is the main disease mechanism in atypical HUS, low C3 and normal C4 serum levels are seldom detected in these patients, thus limiting the diagnostic usefulness of these simple and largely available tests. The identification of mutations in the genes encoding complement proteins (factor H, factor I, membrane cofactor protein (MCP) and other rarer proteins) is not necessary to diagnose atypical HUS, not only because gene analysis is time-consuming and of limited availability, but also because no mutation is identified in at least half of cases.

Table 46.4 Clinical conditions presenting as thrombotic microangiopathies (TMAs) requiring the differential diagnosis with TTP and HUS.

- Disseminated intravascular coagulation
- Pre-eclampsia/HELLP syndrome
- Scleroderma/systemic lupus erythematosus
- Catastrophic antiphospholipid antibody syndrome
- Disseminated malignancy

Table 46.5 Components of the PLASMIC score.

	Points
Platelet count <30.000	1
Bilirubin >2 mg/dL, reticulocytes >2.5%, undetectable haptoglobin	1
No active cancer	1
No history of organ transplantation	1
MCV <90 fL	1
INR <1.6	1
Creatinine <2 mg/dL	1

Score 6–7: high risk of TTP.
Score 5: intermediate TTP risk.
Score 0–4: low TTP risk.

Differential diagnosis of TTP from other thrombotic microangiopathies

Acquired TMAs can also be observed in syndromes such as disseminated intravascular coagulation (DIC), the HELLP (*H*aemolysis, *E*levated *L*iver enzymes and *L*ow *P*latelets) syndrome in pregnant women, the catastrophic antiphospholipid antibody syndrome, pre-eclampsia and eclampsia, cancer and stem cell transplantation.

DIC can be distinguished from TTP by more markedly increased plasma levels of D-dimer, hypofibrinogenaemia and prolonged prothrombin and activated partial thromboplastin times. Because pregnancy is one of the most frequent clinical associations of TTP, differential diagnosis also involves other pregnancy-related TMAs such as pre-eclampsia and eclampsia. In the latter, hypertension is more frequent and renal damage is not as severe, whereas the degree of anaemia and thrombocytopenia is usually more severe in TTP. Tests of coagulation and fibrinolysis are usually abnormal in pre-eclampsia or eclampsia, albeit less markedly than in DIC. Very high serum transaminases differentiate HELLP from TTP in pregnancy. Disorders such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma and the so-called catastrophic antiphospholipid antibody syndrome that sometimes present acutely with widespread microvascular thrombosis, mechanical haemolytic anaemia and consumptive thrombocytopenia, may be distinguished from TTP because laboratory tests such as antinuclear and antiphospholipid antibodies and the detection of the lupus anticoagulant give positive results. Another element of differential diagnosis is the presence of venous and arterial thromboses in large blood vessels, at variance with TTP.

Evans syndrome, due to the concomitant presence of antiplatelet and antierythrocyte autoantibodies, is distinguished by a positive Coombs test, lack of schistocytes and the usual absence of end-organ ischaemic symptoms. At variance with TTP thrombus formation in large venous or arterial vessels are frequent features of heparin-induced thrombocytopenia (HIT) and the recently described Vaccine-Induced Thrombotic Thrombocytopenia (VITT), but TTP can be differentiated not only by lack of exposure to heparin or anti-SARS-CoV-2 vaccines but also by the presence of haemolytic anaemia and schistocytosis. Disseminated malignancy is sometimes associated with consumptive thrombocytopenia and haemolytic anaemia. Until further investigations exclude or confirm the presence of metastatic cancer, it is not easy to distinguish TTP from this TMA. ADAMTS13 plasma levels have been measured in these TMAs and several other unrelated conditions and physiological states, such as the neonatal state, pregnancy, localised and metastatic cancer, liver cirrhosis, inflammatory states, postoperative period, uraemia, sepsis and autoimmune diseases. In the majority of these instances ADAMTS13 activity was lower in patients than in controls but, in contrast to TTP, at plasma levels of 10% or more.

Natural history

In approximately two-thirds of cases TTP occurs only once (acute sporadic TTP). However, in at least one-third, the disease recurs at varying time intervals after remission from the acute episode. Recurrence is not easily predictable but occurs more often during the first year and in patients who have persistently low levels of ADAMTS13 during regular monitoring after first remission (Table 46.3). Recurrences may comprise single or several episodes and usually present with less severe clinical manifestations, perhaps because patients have become alert to the first signs and symptoms of their disease.

Treatment

The efficacious management of TTP began with the serendipitous observation that plasma infusion improved the clinical course. Plasma exchange (PEX) subsequently appeared to be more effective than infusion. In 1991, the results of a seminal randomised clinical trial established the greater efficacy of PEX over infusion, the clinical response rate being 78% in the former compared with 49% in the latter (mortality rates, 22% and 37%, respectively). Only after the discovery of the mechanistic role of VWF and its cleaving protease ADAMTS13 have these empirical treatments found a rationale. PEX acts by removing circulating disease-causing autoantibodies, the most frequent mechanism of acquired TTP but also by replacing the protease in cases of congenital and acquired deficiency.

In congenital TTP, ADAMTS13 replacement therapy is the essence of management and can be achieved by infusion of fresh-frozen plasma and also of some virally inactivated, intermediate-purity plasma factor VIII-VWF concentrates that contain relatively large amounts of ADAMTS13, such as 8Y (BPL; BioProducts Laboratory, Elstree, Herts) and KoateVR-DVI (Kedrion Biopharma, USA). The advantage of these products is that they can be administered with a small infusion volume in the outpatient or home setting (6). BPL 8Y has been used with success at a dosage of 15–30 U/kg, although there is no standardised content of ADAMTS13 in this or other plasma-derived products. Despite the fact that ADAMTS13 has a half-life of 2–3 days, the clinical effect of plasma (20–30 mL/kg) or 8Y are such that infusions every 2 weeks are usually sufficient to supply enough ADAMTS13 to maintain low but measurable plasma levels of the protease and avoid platelet consumption and related clinical symptoms. Since recurrence is not a constant finding and is not accurately predictable, it is not firmly established which patients should undergo regular prophylaxis and which peak and trough

ADAMTS13 levels are necessary, either to attain remission of the acute episode or to prevent recurrences. There is as yet no definite evidence that patients with congenital ADAMTS13 deficiency treated with plasma or plasma products develop alloantibodies toward the protease. The recent ISTH guidelines on treatment of congenital TTP suggest prophylactic plasma infusion or a watch and wait strategy (a conditional recommendation in the context of very low level of certainty) and suggest against the use of FVIII-VWF concentrate versus a watch and wait strategy (a conditional recommendation in the context of very low certainty). A recombinant preparation of ADAMTS13 improves defective proteolysis of VWF *in vitro*, but this product is still in a Phase 3 clinical trial and not yet licensed for clinical use as replacement therapy in congenital TTP.

Acquired TTP. A simple scheme summarising the steps to be followed in the acute management of acquired immune-mediated TTP is in Table 46.6. Treatment with PEX should be initiated as soon as the clinical diagnosis of TTP is suspected. However, plasma infusion is still the only therapy with nearly global availability and critically ill TTP patients are often first admitted to hospitals without facilities for PEX. In these circumstances, daily infusion of large amounts of fresh-frozen plasma (FFP) (30 mL/kg) should be promptly started (Table 46.6), implementing at the same time measures to perform PEX. Patients are best admitted to intensive care units, where coma, seizures and such severe side-effects of PEX as congestive heart failure, catheter-related bleeding, thrombosis and infections can be optimally managed. At least 1–1.5 plasma volumes should be exchanged daily until platelets rise and a clinical response is obtained, defined as platelet count $>150 \times 10^9$ IL sustained for 3–4 days, Daily treatment with PEX must be continued for at least 3–4 days even after attaining a clinical response (Table 46.6). Tapering PEX (over 1–2 weeks) seems to reduce the risk of early relapse. Even though a clinical response is usually achieved after 5–10 days, daily PEX for up to 1 month is sometimes necessary. Fresh-frozen plasma treated with virus-inactivation methods (e.g. solvent/detergent) should be preferred if possible in the context of PEX, because it decreases the risk of infections associated with patient exposure to plasma from a large number of donors.

Table 46.6 Steps in the acute management of acquired, immune mediated TTP.

- Clinical suspicion (based on severe thrombocytopenia, mechanical haemolytic anaemia, presence of schistocytes in the blood film, high serum LDH) and the use of the French and PLASMIC scores.
- Plasma exchange (PEX) should be started as soon as possible.
- Daily plasma exchanges (1–1.5 plasma volumes) until platelets are higher than 150×10^9/L for at least 3–4 days and LDH is normal.
- Immunomodulation: prednisone 1–2 mg/kg for at least 15 days, then tapering over 30 days.
- Immunomodulation anti-CD20 (rituximab, 375 mg/m^2 weekly for 4 weeks)
- Anti-VWF caplacizumab: 10 mg upfront intravenously, then 10 mg/day subcutaneously for at least 20 days.

The immune-mediated mechanism of acquired TTP must be tackled by means of immunomodulating agents but always in association to PEX, which is the primary weapon. Corticosteroids are usually the first choice at the time of acute presentation (Table 46.6). A recommendation from the ISTH guidelines in the context of very low certainty enlists the addition of corticosteroids over PEX alone for patients with immune mediated TTP experiencing a first acute event. For those patients experiencing a relapse, the ISTH guidelines recommend addition of corticosteroids to PEX over PEX alone (a recommendation in the context of very low level of certainty).

Another immunomodulating medicine currently recommended as an adjunct to standard PEX in the acute management of immune-mediated TTP is the anti-CD20 humanised monoclonal antibody rituximab, that blocks the production of anti-ADAMTS13 antibodies through the depletion of B lymphocytes. Rituximab is generally administered once weekly at 375 mg/m^2 for four consecutive weeks immediately after the end of each PEX procedure (Table 46.6). The plasma levels of ADAMTS13 need to be checked after 2 weeks and if levels are not satisfactorily increased prolongation of this therapy until 6 weeks should be considered (personal experience). For patients experiencing their first acute event or relapse, ISTH guidelines suggest the addition of rituximab to corticosteroids and PEX over corticosteroids and PEX alone (a conditional recommendation in the context of very low level of certainty). For prophylaxis of patients in remission who still have low plasma ADAMTS13 activity but no clinical signs/symptoms, a conditional recommendation in the context of very low level of certainty suggests the preferred use of rituximab over its non-use. Many other immunomodulatory agents used in the past (vincristine, cyclosporin, cyclophosphamide) are no longer considered valid options due to the risk of adverse effects coupled with less efficacy than corticosteroids and rituximab. The clinical efficacy of antiplatelet agents in TTP is unproven and platelet transfusion is generally ineffective.

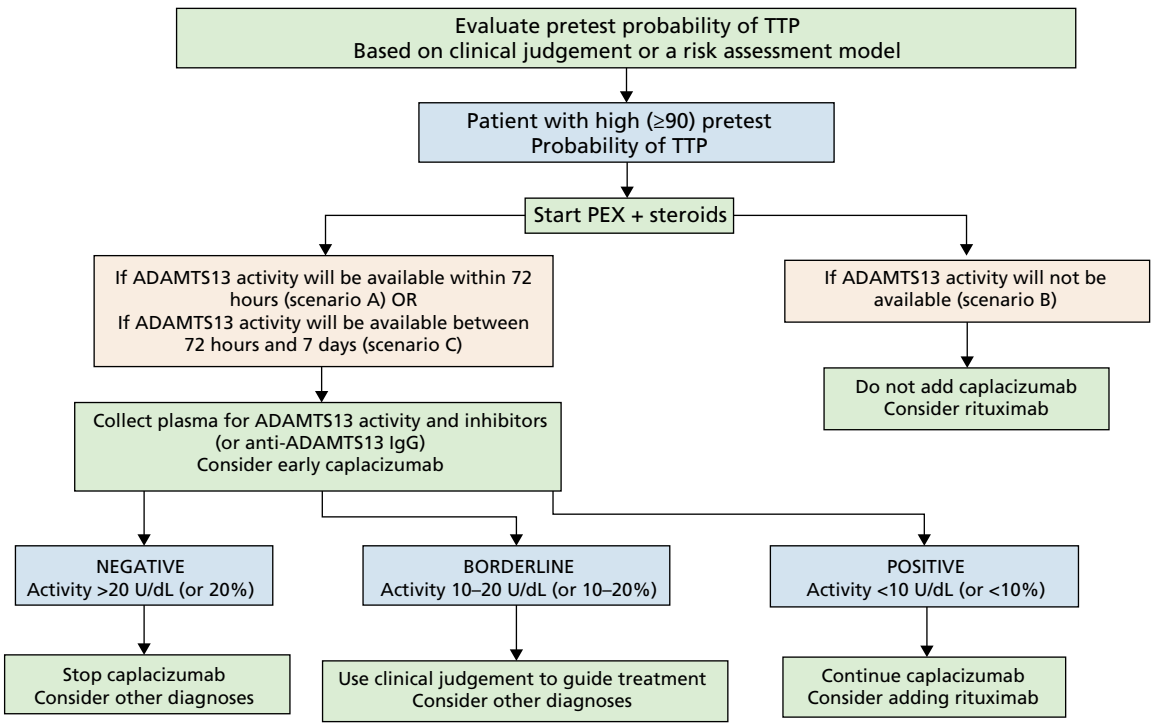
A new therapeutic agent acting upon the VWF disease pathway has been licensed in 2018 by the FDA and EMA for the management of acute TTP. Caplacizumab is a first-in-class humanised nanobody which binds the A1 domain of VWF and prevents platelet aggregation and thrombus

formation in the microcirculation by blocking the interaction between ultra-large VWF and the platelet glycoprotein Ib-IX-V. This agent, which targets VWF and thus does not address the underlying autoimmunity and the anti-ADAMTS13 autoantibody, gave good clinical results as an adjunct to PEX and immunomodulation in the frame of the two randomised trials TITAN and HERCULES. A faster reversal of thrombocytopenia (within a median time of 3 days) was obtained with a 74% reduction of a combined endpoint (death, recurrence and major thromboembolic events) and also a shorter duration of PEX and hospitalisation. The approved daily dose of caplacizumab is 10 mg according to EMA and 11 mg for FDA, first intravenously and then subcutaneously (Table 46.6), and it should be continued until ADAMTS13 levels are kept above 20% for at least 2 weeks. According to the ISTH guidelines, caplacizumab should be preferred over not using this drug in those patients with immune-mediated TTP who experience an acute event (first event or relapse). The data informing this recommendation were of moderate certainty.

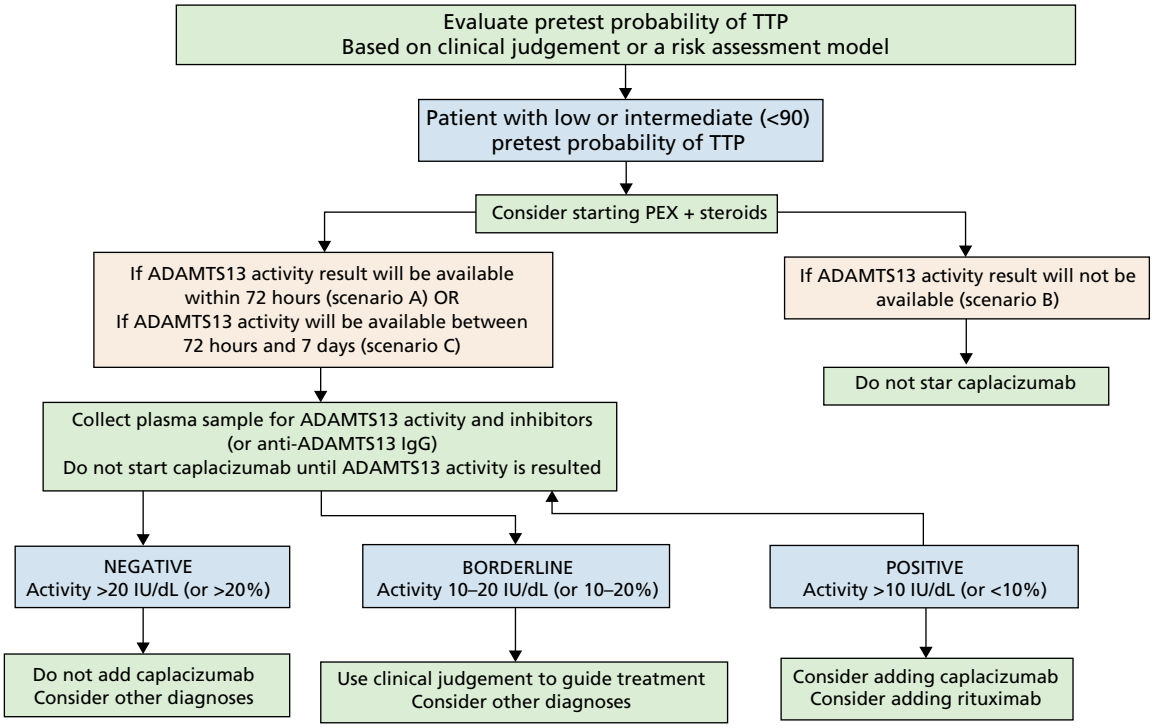
The benefit of caplacizumab is accrued if started in the early phase of an acute TTP event i.e. at the time when a diagnosis is confirmed. Therefore, caplacizumab use should be conditional on the capacity to accurately identify patients with a high clinical suspicion of TTP, evidence of severe deficiency of plasma ADAMTS13 activity and presence of anti-ADAMTS13 IgG. In settings characterised by no access to *ADAMTS13* testing, caplacizumab should not be used, regardless of the pretest probability of TTP. Major hurdles to its widespread use are cost and accessibility in various parts of the world. Moreover, caplacizumab should only be given under the guidance of clinicians experienced in TTP and with the required level of knowledge on the use of this drug.

Out of the acute phase, some patients with immune mediated TTP need to continue immunomodulation to prevent recurrences. In the remission phase, this approach is usually implemented in those who had recurrences (usually after intervals ranging from as little as 1 month from the first acute episode) but also upfront after first remission in the presence of clinical conditions associated with a high risk of recurrence (pregnancy, elective surgery, active autoimmune disorders) and with evidence of persistent severe deficiency of ADAMTS13 and presence of anti-ADAMTS13 antibodies. In these cases, the first choice is usually rituximab or lower doses of corticosteroids than those used in the acute phase. Second-choice drugs in unresponsive patients are cyclosporine or azathioprine.

To sum up, the enclosed flow chart summarises the management of patients with immune-mediated TTP according to the ISTH guidelines. In settings characterised by a timely access to *ADAMTS13* testing and for patients with a high clinical suspicion (≥90% pretest probability) of immune-mediated TTP (as based on clinical assessment or a formal clinical risk assessment scores), the following strategy is suggested:



In settings with a timely access to *ADAMTS13* testing but for patients with intermediate or low clinical suspicion of immune-mediated TTP (e.g. based on clinical assessment or a formal clinical risk assessment scores), the following strategy is suggested:



Haemolytic uraemic syndrome

The most typical and frequent forms of HUS are acquired and occur acutely and sporadically, particularly in infants and young children, after gastrointestinal infections associated with bacteria as Escherichia coli that produce the Shiga-like toxin, so that it is called *S*higa-like *T*oxin-producing *E*scherichia *C*oli (STEC) HUS. More seldom, 10% of HUS are cases of the so-called atypical HUS (aHUS) that can be familial, due to the inherited dysfunction of complement proteins and may have a sporadic or chronic recurrent course. It occurs mainly in infants or young children, but at least one-third of cases develop in adulthood, often in association with triggers such as delivery or drug intake.

STEC HUS

This acute syndrome almost always presents as a single sporadic episode heralded by bloody diarrhoea from 2 days to 2 weeks before the onset of symptoms. Besides consumptive thrombocytopenia and mechanical haemolytic anaemia in common with TTP, the most typical symptoms are severe renal failure with oligo-anuria. Serum creatinine is much more abnormal than in TTP, thrombocytopenia is usually less severe. Signs of compensated DIC, with relatively high plasma levels of D-dimer, are another distinctive feature. Neurological involvement is less common than in TTP, but coma and seizures may occur in association with uraemia and severe hypertension.

Aetiology

The most common bacterial agents that cause the prodromal gastrointestinal infection are *Escherichia coli* type 0157:H7 or O:104 or, more seldom, *Shigella dysenteriae* serotype I. These and other less frequently involved infectious agents such as pneumococci produce exotoxins called verocytotoxin, Shiga toxin or Shiga-like toxins, that can be detected on stool samples by culture methods or polymerase chain reaction (PCR) assays. These toxins, after absorption from the gastrointestinal tract into the blood, bind to the glycosphingolipid membrane receptor globotriaosylceramide, which is particularly dense in the kidney glomerular capillary endothelial cells. The toxin–receptor complex is endocytosed and thereby causes cytolysis and extensive endothelial swelling and desquamation, which in turn engenders the formation of microthrombi in the renal microvasculature, mainly composed of platelets but also rich in fibrin (Figure 46.3).

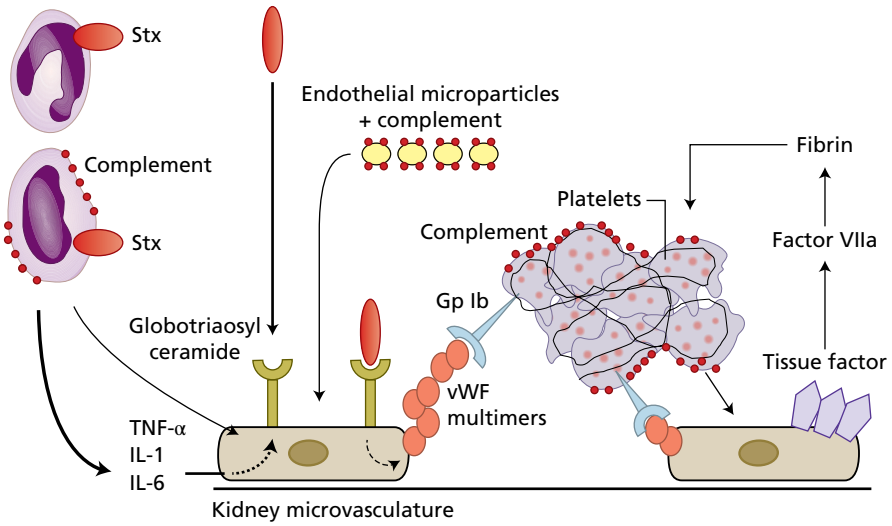


Figure 46.3 Pathophysiology of STEC HUS. Verocytotoxin, Shiga toxin or Shiga-like toxins, after absorption from the gastrointestinal tract into the blood, bind to the glycosphingolipid membrane receptor globotriaosylceramide on the glomerular capillary endothelial cells. The toxin–receptor complex is endocytosed and causes cytolysis, endothelial swelling and desquamation, which leads to massive formation of microthrombi in the renal microvasculature, mainly composed of platelets but also rich in fibrin. Gp Ib: platelet glycoprotein Ib; IL: interleukin; Stx: Shiga toxin; TNF: tumour necrosis factor.

Pathology and pathogenesis

The pathology of STEC-HUS is characterised by more extensive endothelial damage than TTP but less VWF and more fibrin are detected in microvascular thrombi. Plasma VWF levels are often very high but ultra-large multimers are not detectable during the acute phase of the disease, perhaps because these multimers leak in excess into plasma from damaged endothelial cells bind avidly to GPIb on the platelet membrane and are thus removed from plasma. As forementioned, ADAMTS13 is normal or only mildly reduced in the plasma of these patients (Table 46.3), this finding at variance with TTP being crucial for the differential diagnosis.

Natural history

In the great majority of cases, STEC-HUS is a self-limiting disease, with little tendency to early or late relapses. The acute episode is often accompanied by severe renal failure, so that haemodialysis may be required. Residual renal dysfunction is common after the acute episode and end-stage renal disease requiring maintenance dialysis or kidney transplantation is needed in approximately one-quarter of patients.

Treatment of STEC HUS

During the diarrhoeal prodrome, antimotility agents are contraindicated, because they favour the persistence of bacterial toxins in the gastrointestinal lumen and their passage into blood. The role of antibiotics is controversial, but in practice, they are often used, with a strong indication at least for patients with evidence or suspicion of sepsis or *Shigella dysenteriae* infection. In addition, the optimal management of STEC HUS includes intravenous hydration and monitoring renal function. In cases of severe renal failure, early institution of dialysis is essential in order to reduce sequelae and death. PEX is not recommended in patients with STEC HUS. A possible forthcoming approach to prevention is vaccination against vero/Shiga toxins in children living in endemic areas.

Atypical HUS

The term atypical HUS (aHUS) is used for TMAs characterised by severe renal failure often associated with the dysregulation of the alternative complement pathway. Given the key role of complement in aHUS, the term complement-mediated HUS (cHUS) is now more often preferred. In the disease, there is an excessive activation of C3 convertase, its partial consumption and production of C3a and C3b, resulting in increased formation of the C5b-9 terminal complement complex (also called the membrane-attack complex [MAC]) that directly damages vascular endothelial cells. C3a and C3b also activate other cells, such as platelets and leucocytes, causing inflammation and thrombosis (Figure 46.4). In aHUS, kidney glomeruli are particularly affected due to the formation of microvascular thrombi. However, microvascular thrombosis may occur almost everywhere but particularly in organs such as the brain, lung and gastrointestinal tract. The acute attacks can be triggered by infections, pregnancy, drugs or trauma in the presence of dysregulation of complement factors and inhibitors, mainly but not exclusively of genetic origin. Approximately half of the patients have loss-of-function mutations of the genes encoding complement inhibitors (factor H, factor I and membrane cofactor protein [MCP]) or gain-of-function mutations in the genes encoding complement activators (complement component 3 [C3] and factor B) (Table 46.7) resulting in excessive C3 activation with the consequences described above. The type of mutation impacts prognosis, patients with mutations in the factor H gene having the poorest prognosis in terms of short- and long-term

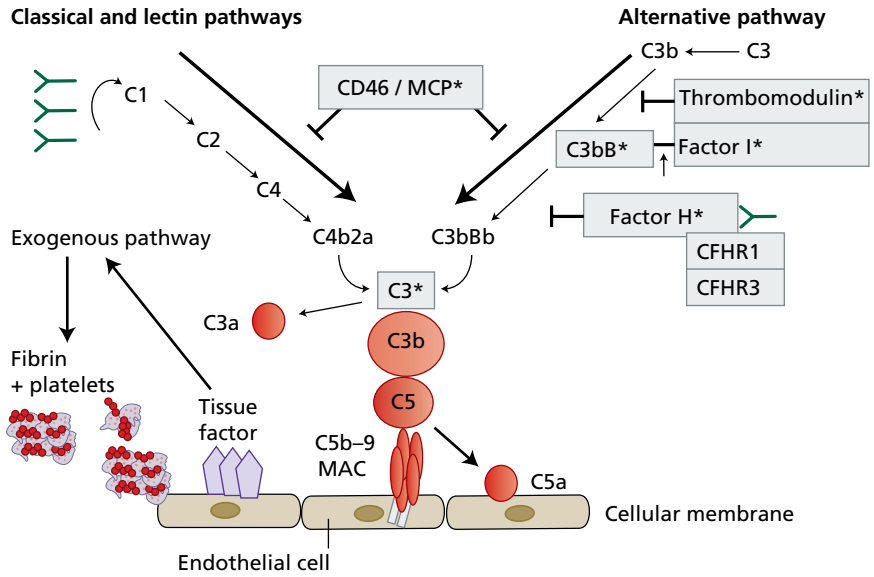


Figure 46.4 Pathophysiology of aHUS. An excessive activation of C3 convertase and its partial consumption with production of C3a and C3b results in the increased formation of the C5b-9 terminal complement complex (also called the membrane-attack complex [MAC]), that in turn causes the damage of vascular endothelial cells. C3a and C3b also activate platelets and leucocytes, causing inflammation and thrombosis. CFHR, complement factor H-related; MAC, membrane attack complex; MCP, membrane cofactor protein. *mutations are reported in genes encoding these complement factors.

Table 46.7 Main complement factors and regulators with their function and frequency of alteration.

Complement factor or antibody	Function	Percentage frequency of gene mutations/antibodies
CFH	• Recognises C3 and mediates cofactor activity for CFI • Accelerates the decay of C3 convertase	20–30
CFI	• Plasma serine protease • Cleaves C3b into inactive C3b	4–10
C3	• Its activation is required for both classical and alternative complement activation pathways • Its proteolytic cleavage produces C3a and C3b; C3a is an anaphylatoxin and the precursor of some cytokines and C3b serves as an opsonising agent	5–10
Thrombomodulin	• Transmembrane protein • Involved in the generation of TAFI, which cleaves C3 and C5a	5
CFB	• Plasma serine protease • Its proteolytic cleavage produces the noncatalytic chain Ba and the catalytic subunit Bb • The active subunit Bb associates with C3b to form the alternative pathway C3 convertase • Bb is involved in the proliferation of preactivated B lymphocytes, while Ba inhibits their proliferation	1–2
MCP	• Integral transmembrane protein that binds C3b and serves as a cofactor for CFI	10–15
CFH antibodies		3–6

CFB, complement factor B; CFH, complement factor H; CFI, complement factor I; MCP, membrane cofactor protein; TAFI: thrombin activatable fibrinolysis inhibitor.

renal function and relapse risk, even after kidney transplantation (see below). On the other hand, patients with MCP mutations have a better prognosis. A few cases of aHUS have also been described in association with the development of acquired autoantibodies against factor H that can be of the IgG class when associated with genetic abnormalities of the factor H-related antigens or of the IgM class in absence of such abnormalities. Given that nearly half of patients with aHUS have no identified complement abnormalities, much remains to be learned pertaining to disease mechanisms and causes other than complement defects may be the culprit. Other TMAs with a clinical presentation of acute kidney injury may be: (i) metabolism-mediated, such as homozygous mutations in the gene encoding the methylmalonic aciduria and homocystinuria type C (MMACHC) protein; (ii) haemostasis-mediated, such as homozygous mutation in the genes encoding thrombomodulin, plasminogen or diacylglycerol kinase ε [DGKE]); (iii) drug-mediated, by an immune reaction (quinine) or a toxic reaction (calcineurin inhibitors such as cyclosporine or tacrolimus).

Laboratory diagnosis

As mentioned above, low serum C3 levels are a simple and widely available method for diagnosing affected patients, but more than 50% of those diagnosed with aHUS have normal levels. Similarly, mutations in complement proteins are important but not sufficient in the development of aHUS, because carriers are often asymptomatic and actual disease precipitation requires triggering factors such as infections, surgery, pregnancy or immune-mediated diseases that activate the complement alternative pathway. It must be reiterated that for the diagnosis it is of paramount importance to measure ADAMTS13 activity to rule out TTP when higher than 10% and look for E. coli-producing Shiga-like toxin in stool samples to rule out STEC HUS if negative. While ADAMTS13 results are awaited, a platelet count >30000/mm^3 and/or a serum creatinine level >2.0 mg/dL strongly suggest a diagnosis of aHUS (Figure 46.5).

Natural history

Until recently HUS was a disease with a poor prognosis with a fatality rate of between 10 and 20% and rate of progression to end-stage renal insufficiency of 50% or more despite the use of PEX. aHUS is often a chronic ongoing systemic disease, because even after recovery from the acute illness patients may suffer from additional episodes if exposed to clinical triggers (infection, surgery, pregnancy) owing to their persistent inability to regulate complement activity.

Treatment

Until the first decade of the 2000s, aHUS was treated with plasma therapy, mainly PEX but also infusion only. The rationale of this approach was the replacement of deficient or dysfunctional complement proteins, but also the removal of activated complement factors and anti-factor H autoantibodies when present. Plasma therapy had to be often combined with haemodialysis to control oligo-anuria and uraemia. Despite poor outcomes (10–20% case fatality rate and 50% or higher rate of progression to stage renal disease) plasma infusion or PEX still remain important treatment weapons. The response to PEX is typically re-assessed at days 2–3, with the goal to establish whether or not there is improvement of anaemia and thrombocytopenia, as well as improvement or at least stabilisation of end-organ damage. In the few patients responding optimally to PEX, this therapy is continued daily until normalisation of surrogate markers of ongoing microthrombotic disease such as platelet count, haemoglobin and LDH and then stopped without prolonged tapering. Markers of kidney damage (high serum creatinine and proteinuria) and neurologic findings and symptoms when present and persistent are of high significance for the clinical management of these patients. A poor response to PEX can be defined as lack of haematological response, but also by worsening kidney insufficiency despite daily PEX. In these cases, PEX should be discontinued and therapy with eculizumab considered.

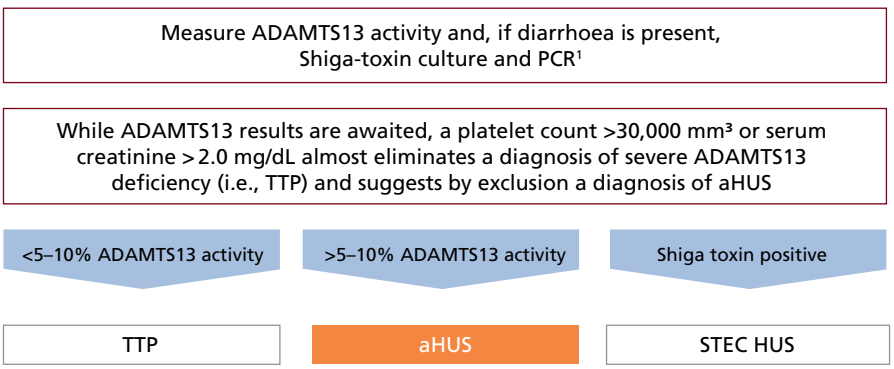


Figure 46.5 Differential diagnosis of patients with a suspected aHUS, TTP or STEC HUS.

Eculizumab, a humanised monoclonal antibody approved in 2011 for the treatment of aHUS, binds to complement protein C5 and blocks the formation of the end product membrane attack complex and thus stands as an effective life- and kidney-sparing therapy for patients with aHUS. This product has been prospectively evaluated for efficacy and safety in aHUS patients with a broad spectrum of disease features (those recently diagnosed or with long disease duration but also those who had already undergone transplant or on continuous dialysis). Therapy often resulted not only in haematologic improvement but also and most importantly in the marked improvement of renal function, even in cases with advanced disease. There is evidence that a rapid initiation of therapy with eculizumab is associated with a greater improvement in renal function in aHUS patients. However, the initiation of eculizumab treatment prior to a window of 2–3 days of PEX is not advised when there are difficulties in differentiating with certainty aHUS from TTP at the time of presentation (Figure 46.5), but eculizumab is recommended as first-line therapy when the diagnosis of aHUS is known at the time of acute presentation (such as, for instance, for recurrences).

Even though eculizumab is usually well tolerated, all patients should receive a meningococcal vaccine owing to the increased risk to acquire this infection. Intravenous eculizumab is initiated at a dose of 900 mg weekly for the first 4 consecutive weeks and then, beginning on week 5, maintenance therapy is administered every other week at a dose of 1200 mg, usually for an indefinite treatment period. These recommended dosing schedules are extremely costly. However, indefinite therapy with eculizumab can be tailored by monitoring global complement tests in order to determine the minimal effective dose to maintain the disease in remission, but with a substantial saving of costs. Attempts have been made to discontinue this therapy in small series of patients but no general recommendation beyond individualisation can be currently offered. Eculizumab is often associated with dramatic clinical responses and, most importantly, with such a high degree of recovery of renal function that at least 80% of patients can discontinue dialysis, in contrast with the previous gloomy picture when most patients died or progressed to end-stage renal disease despite PEX. It is of extreme importance to avoid permanent dialysis and renal transplantation, because the results of the latter are often poor and appear to correlate with the cause of complement dysregulation. For instance, the disease did not recur in the transplanted kidneys of patients with *MCP* gene mutations, presumably because the new kidney produces protective levels of endogenous MCP. In contrast, kidney transplantation alone did not correct the deficiency of complement factors I and H produced by the liver, so that in patients with factor H or factor I gene mutations, outcome was poor owing to disease recurrence. However, an approach as formidable as combined liver and kidney transplantation may lead to a favourable long-term outcome for recurrent HUS associated with *factor H* gene mutations.

A long-acting C5 inhibitor with the name ravulizumab has been recently developed through minimal modifications of eculizumab and approved for the treatment of aHUS both in the US and in Europe. Clinical data in paediatric patients with aHUS have shown that ravulizumab can be safely administered and the dosing interval prolongation up to 4–8 weeks, due to a half-life 4 times longer than that of eculizumab.

Concluding remarks

In the last few years, TTP and HUS, previously known to clinicians and pathologists for their dire consequences, have witnessed spectacular improvements owing to increased mechanistic knowledge and development of new specific diagnostic criteria particularly for TTP, whereas atypical HUS still remains a diagnosis of exclusion. Most importantly, improved therapeutic options led to a marked decrease in mortality and morbidity, particularly for atypical HUS following the availability of eculizumab. In TTP, the pivotal role of VWF and the deficiency or dysfunction of its major proteolytic enzyme ADAMTS13 in inducing microvascular platelet-rich thrombi is evident. The development of laboratory methods to measure ADAMTS13 in plasma demonstrated that bona fide TTP cases have very low or unmeasurable levels of this protease. Trigger(s) are usually needed for the occurrence of full-blown TTP, because patients with congenital and immune-mediated, acquired deficiency of ADAMTS13 may not manifest clinical disease for long periods of time. The clinicians confronted with the treatment of these seriously ill patients should be cognisant that plasma therapy, mainly plasma exchange and immunomodulation with rituximab remain the treatment of choice for patients with clinically diagnosed TTP, but the anti-VWF nanobody caplacizumab is a useful adjunct to the PEX/rituximab approach. An important not completely resolved therapeutic problem is the secondary prevention of the chronic recurrent forms. Various immunomodulatory treatments have been tried with promising results, particularly rituximab.

STEC-HUS is now well characterised from an aetiological standpoint, so that it can be specifically recognised and properly treated. The dire consequences of atypical HUS, that is still lacking specific diagnostic tests, are now dramatically improved by the availability of a complement inhibition therapy with the humanised monoclonal antibody eculizumab, that is able to induce not only a dramatic therapeutic response in many patients unresponsive to plasma therapy but also the recovery of renal function, in contrast with the poor prognosis for patients treated only with plasma-based therapy. However, long-term complement inhibition comes with an immense financial burden, the inconvenience of frequent infusions and the small but real risk of meningococcal infection associated with indefinite

therapy. The problem of frequent infusions has been addressed by lengthening the dosing interval of eculizumab based on the measurement of complement activation markers and by making clinically available an extended half-life C5 inhibitor. On the other hand, the efficacy and safety of eculizumab discontinuation remains uncertain and this choice must be accurately individualised.

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CHAPTER 47

Antithrombotic agents

47

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Introduction

Antithrombotic drugs used for the prevention and treatment of thrombosis include antiplatelet drugs, which inhibit platelet activation and aggregation; anticoagulants, which attenuate coagulation; and fibrinolytic agents, which induce fibrin degradation. With the predominance of platelets in arterial thrombi, strategies to prevent or treat arterial thrombosis focus mainly on antiplatelet agents, although anticoagulants and fibrinolytic agents are often used in the acute setting. The addition of low-dose rivaroxaban to dual antiplatelet therapy with aspirin plus clopidogrel or to single antiplatelet therapy with aspirin alone reduces the risk of recurrent ischaemic events in patients with an acute coronary syndrome (ACS) or in those with stable coronary or peripheral artery disease. These observations highlight the utility of anticoagulants on top of antiplatelet agents, so-called dual pathway inhibition, for secondary prevention in patients with atherosclerosis.

Anticoagulants are the mainstay for the prevention and treatment of venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE). Antiplatelet drugs are less effective than anticoagulants for VTE prevention because of the limited platelet content of venous thrombi. Selected patients with VTE benefit from fibrinolytic therapy such as those with severe PE or extensive proximal DVT.

This chapter describes the antiplatelet drugs and anticoagulants in common use.

Antiplatelet Agents

The antiplatelet agents include aspirin, dipyridamole, inhibitors of $P2Y_{12}$, glycoprotein (GP) IIb/IIIa antagonists, and vorapaxar. Their sites of action are illustrated in Figure 47.1.

Aspirin

Mechanism of action: Aspirin produces its antithrombotic effect by irreversibly acetylating and inhibiting platelet cyclooxygenase-1 (COX-1), a critical enzyme in the biosynthesis of thromboxane A_2. At higher doses, aspirin also inhibits COX-2, an inducible COX isoform found in endothelial and inflammatory cells. In endothelial cells, COX-2 initiates the synthesis of prostacyclin, a potent vasodilator, and inhibitor of platelet activation that antagonises the effects of thromboxane A_2. Because acetylation of COX-1 by aspirin is irreversible and because anucleate platelets cannot synthesise more of the enzyme, COX-1 activity is lost for the platelet lifetime, which is 8–10 days. About 10% to 12% of circulating platelets are replaced every day; however, the recovery of platelet function is faster than that predicted by the rate of platelet turnover because aspirin-treated platelets can respond to TXA_2 generated by newly produced platelets. Consequently, stopping aspirin for 5 to 7 days is sufficient to restore normal platelet function.

Indications: Aspirin is routinely used for secondary prevention in patients with ischaemic stroke, ACS, or peripheral artery disease where it reduces the risk of recurrent ischaemic events by about 20%. Aspirin is no longer recommended for primary prophylaxis except in the highest-risk patients.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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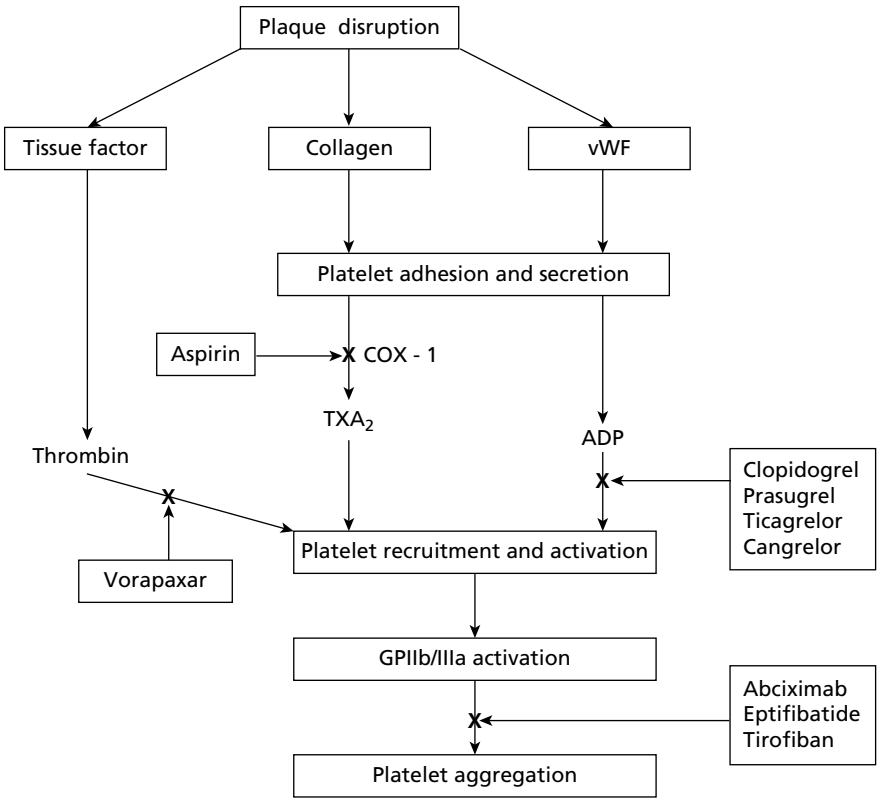

Figure 47.1 Sites of action of antiplatelet drugs. Aspirin inhibits the synthesis of thromboxane A_2 (TXA_2) by irreversibly acetylating cyclo-oxygenase 1 (COX-1). The reduced release of TXA_2 attenuates platelet activation and recruitment to the site of vascular injury. Clopidogrel, and prasugrel irreversibly block $P2Y_{12}$, a key ADP receptor on the platelet surface; cangrelor and ticagrelor are reversible inhibitors of $P2Y_{12}$. Abciximab, eptifibatide and tirofiban inhibit the final common pathway of platelet aggregation by blocking binding of fibrinogen and von Willebrand factor (vWF) to activated GP IIb/IIIa. Vorapaxar inhibits thrombin-mediated platelet activation by targeting protease-activated receptor-1 (PAR-1), the major thrombin receptor on platelets.

Dosing: At doses of 75–100 mg/day, aspirin abolishes TXA_2 synthesis without significant inhibition of COX-2 and subsequent impairment of prostacyclin synthesis.

Side Effects: The most common side effects are gastrointestinal, which range from dyspepsia to erosive gastritis or peptic ulcers with bleeding and perforation. The use of enteric-coated or buffered aspirin in place of plain aspirin does not eliminate these side effects. The risk for major bleeding with aspirin is 1% to 3% per year; the risk is increased with the concomitant use of anticoagulants.

Aspirin allergy characterised by bronchospasm occurs in about 0.3% of the general population and is more common in patients with chronic urticaria or asthma, particularly those with nasal polyps or chronic rhinitis. Aspirin overdose is associated with hepatic and renal toxicity.

Aspirin resistance: The term *aspirin resistance* is used to describe both clinical and laboratory phenomena. Clinical aspirin resistance is defined as the failure of aspirin to protect patients from ischaemic vascular events, a diagnosis that can only be made after such events occur. This retrospective diagnosis provides no opportunity to modify therapy nor is it realistic to expect aspirin, which selectively blocks thromboxane A_2 biosynthesis, to prevent all vascular events. The biochemical definition of aspirin resistance involves the failure of the drug to inhibit thromboxane A_2 synthesis or arachidonic acid-induced platelet aggregation. Potential mechanisms for such resistance include poor adherence, reduced absorption of enteric-coated aspirin, thromboxane A_2 generation via COX-1 independent pathways, and genetic polymorphisms in *COX-1*. High body weight and increased platelet turnover after surgery or in patients with myeloproliferative disorders are associated with an increased risk for aspirin resistance. Tests used for the diagnosis of biochemical aspirin resistance include quantification of thromboxane B_2, the stable metabolite of thromboxane A_2, in serum or urine, or assessment of arachidonic acid-induced platelet aggregation using light transmission aggregometry or point-of-care devices such as the VerifyNow® system. Tests for aspirin resistance should remain research tools because there is

no evidence that they identify patients at higher risk for recurrent vascular events or that the resistance is reversed with higher doses of aspirin or the addition of other antiplatelet drugs.

Dipyridamole

Mechanism of action: Dipyridamole attenuates platelet aggregation by increasing the intracellular levels of cAMP. This occurs in two ways. First, dipyridamole increases plasma adenosine levels by blocking adenosine reuptake. Adenosine stimulates adenylyl cyclase in platelets leading to increased intracellular cAMP. Second, by reversibly inhibiting platelet phosphodiesterase, dipyridamole prevents cAMP degradation. Inhibition of phosphodiesterase in the vessel wall results in vasodilation by increasing endothelial cell prostacyclin production and by increasing cGMP levels in vascular smooth muscle cells.

Indications: Slow-release dipyridamole in conjunction with aspirin is used for secondary stroke prevention in patients with prior stroke or transient ischaemic attack (TIA). Compared with clopidogrel, this combination was no more effective and was associated with more bleeding and more discontinuations because of side effects. Therefore, dipyridamole is rarely used.

Dosing: The fixed combination, which contains 200 mg of extended-release dipyridamole and 25 mg of aspirin is given twice daily.

Side effects: Dipyridamole produces vasodilation and its major side effects include headache, facial flushing, dizziness, hypotension, and gastrointestinal complaints, symptoms that often subside with continued drug use.

P2Y12 antagonists

P2Y12 is the major ADP receptor on platelets. The P2Y12 antagonists include clopidogrel, prasugrel, ticagrelor, and cangrelor.

Clopidogrel

Mechanism of action: Clopidogrel is a prodrug that requires metabolic activation by cytochrome P450 (CYP) 2C19 to acquire activity. Consequently, its onset of action is delayed unless loading doses are given. The active metabolite has an elimination half-life of about 30 to 60 minutes, but its antiplatelet effect persists for the lifetime of the platelet because it acts by forming an irreversible disulfide bond with the receptor. Therefore, clopidogrel should be held for 5 to 7 days prior to surgery or invasive procedures associated with a high risk of bleeding.

Indications: Clopidogrel is indicated, often in combination with aspirin, for secondary prevention of atherothrombotic events in medically treated patients with ACS. For those undergoing coronary stent implantation, clopidogrel combined with aspirin is recommended for 1 year with at least 3 months of treatment for drug-eluting stents and 1 month for bare-metal stents. Clopidogrel may be added to aspirin for secondary prevention in patients with stroke or TIA; clopidogrel is stopped after 3 to 4 weeks to minimise the risk of bleeding, and patients are continued on aspirin monotherapy. Clopidogrel monotherapy can be used in place of aspirin for secondary prevention in patients with a history of ischaemic stroke, myocardial infarction, or peripheral artery disease.

Dosing: After an initial loading dose of 300 to 600 mg, clopidogrel is given at a dose of 75 mg once daily.

Side effects: The major side effect is bleeding. Concomitant administration of aspirin or an anticoagulant increases the risk of bleeding. Although platelet transfusion can overcome the antiplatelet effects of clopidogrel, it should be used with caution in patients with haemorrhagic stroke.

Clopidogrel resistance: The capacity of clopidogrel to inhibit ADP-induced platelet aggregation varies amongst subjects reflecting, at least in part, genetic polymorphisms in the *CYP2C19*, which is involved in its metabolic activation. Patients with the loss-of-function *CYP2C19*2* allele exhibit reduced platelet inhibition in comparison with those with the wild-type *CYP2C19*1* allele and are at higher risk of cardiovascular events. Up to 25% of Whites, 30% of Blacks, and 50% of Asians carry the loss-of-function allele, which may render them resistant to clopidogrel. Polymorphisms in *CYP3A4* can contribute to reduced metabolic activation of clopidogrel and polymorphisms in *ABCB1* may impair clopidogrel absorption.

Prasugrel

Mechanism of action

Like clopidogrel, prasugrel is a thienopyridine prodrug that requires metabolic activation. Prasugrel is activated more efficiently than clopidogrel, which results in more rapid, greater and more predictable inhibition of ADP-induced platelet aggregation. Therefore, prasugrel overcomes the resistance observed with clopidogrel even in subjects with loss-of-function *CYP2C19*2* alleles. Prasugrel produces irreversible P2Y12 inhibition and should be held for 5–7 days prior to surgery or invasive procedures associated with a high risk of bleeding.

Indications

Prasugrel, administered with aspirin, is indicated for the secondary prevention of ischaemic events in patients with an ACS who are managed with PCI. In this setting, prasugrel is more effective than clopidogrel but is associated with an increased risk of bleeding. Prasugrel is no more effective than clopidogrel in patients with ACS who are managed medically.

Dosing

Prasugrel is given as a loading dose of 60 mg followed by 10 mg once daily. The dose is reduced to 5 mg once daily in those over the age of 75 years or weighing less than 60 kg.

Side-effects

The major side-effect is bleeding, which can be treated with platelet transfusion. Concomitant administration of aspirin or an anticoagulant increases the risk of bleeding

Ticagrelor

Mechanism of action

An orally active agent belonging to the cyclopentyltriazolopyrimidine class, ticagrelor reversibly binds to $P2Y_{12}$ at a location distinct from the ADP binding site and blocks receptor activation via an allosteric mechanism. Because it does not require metabolic activation, ticagrelor has a more rapid onset of action than clopidogrel or prasugrel. When possible, ticagrelor should be stopped 5 days prior to surgery because its offset of action is slow.

Indications

Ticagrelor, together with aspirin, is indicated for secondary prevention of atherothrombotic events in patients with an ACS treated medically or with PCI (with or without stent implantation) or coronary artery bypass graft surgery. It is also indicated for patients with a history of MI at least 1 year ago who are at high risk of recurrent atherothrombotic events.

Dosing

For patients with ACS, a loading dose of 180 mg is followed by 90 mg twice daily for up to 1 year. For patients with a history of MI, the dose is 60 mg twice daily for up to 3 years.

Side-effects

In addition to bleeding, the most common side-effects include dyspnoea, which occurs in up to 15% of patients, and bradyarrhythmia. The dyspnoea is usually self-limited, but drug discontinuation is necessary if the dyspnoea persists or is severe in intensity.

Platelet transfusion is ineffective for ticagrelor reversal because the drug binds to P2Y12 on the transfused platelets. Bentracimab, an antibody fragment that binds ticagrelor and its metabolite with high affinity, is under development for rapid ticagrelor reversal.

Cangrelor

Mechanism of action

Cangrelor is a rapidly acting reversible inhibitor of $P2Y_{12}$. It has an immediate onset of action after intravenous administration, a half-life of 3–5 min, and an offset of action within 1 hour.

Indications

Cangrelor is indicated for patients undergoing PCI who have not received pretreatment with clopidogrel, prasugrel or ticagrelor and are not receiving a GPIIb/IIIa inhibitor.

Dosing

Cangrelor is administered as a 30 μg/kg intravenous bolus before PCI followed by an infusion of 4 μg/kg/min for at least 2 hours or for the duration of PCI, whichever is longer. When transitioning to oral $P2Y_{12}$ inhibitor therapy, ticagrelor can be given at a loading dose of 180 mg at any time during or immediately after the cangrelor infusion. Loading doses of prasugrel or clopidogrel should only be given after the cangrelor infusion is stopped because cangrelor blocks their interaction with $P2Y_{12}$.

Side-effects

The most common side-effect is bleeding.

GPIIb/IIIa antagonists

The GPIIb/IIIa antagonists include abciximab, eptifibatide and tirofiban (Table 47.1)

Mechanism of action

GPIIb/IIIa, which is found on the surface of platelets and megakaryocytes, is the most abundant platelet receptor. GPIIb/IIIa is inactive on resting platelets but undergoes conformational activation upon platelet activation. Once activated, GPIIb/IIIa binds fibrinogen and von Willebrand factor, which then bridge adjacent platelets together to induce platelet aggregation. Because they inhibit the final common pathway of platelet aggregation, GPIIb/IIIa inhibitors are the most potent of the antiplatelet agents.

Table 47.1 Features of GPIIb/IIIa antagonists.

Feature	Abciximab	Eptifibatide	Tirofiban
Description	Fab fragment of humanised mouse monoclonal antibody	Cyclical KGD-containing heptapeptide	Nonpeptidic RGD mimetic
Specific for GP IIb/IIIa	No	Yes	Yes
Plasma half-life	Short (min)	Long (2.5 h)	Long (2.0 h)
Platelet-bound half-life	Long (days)	Short (s)	Short (s)
Renal clearance	No	Yes	Yes

Abciximab, eptifibatide and tirofiban are structurally and pharmacologically distinct. Abciximab is a Fab fragment of a humanised murine monoclonal antibody directed against the activated form of GPIIb/IIIa, whereas eptifibatide and tirofiban are synthetic small molecules. Eptifibatide is a cyclic heptapeptide that competes with fibrinogen for receptor binding because of its Lys-Gly-Asp motif. Tirofiban is a nonpeptidic tyrosine derivative that inhibits fibrinogen and von Willebrand factor binding to GPIIb/IIIa by serving as an Arg-Gly-Asp mimetic.

Abciximab has a plasma half-life of 7 days but can be detected on the surface of platelets for up to 2 weeks. Eptifibatide and tirofiban have plasma half-lives of about 2 hours. Abciximab is cleared by proteolysis, whereas eptifibatide and tirofiban are cleared via the kidneys.

Indications

GPIIb/IIIa antagonists are used in high-risk patients undergoing PCI, particularly those with ST-segment-elevation MI who have not been adequately pretreated with antiplatelet drugs or those requiring rapid platelet inhibition. Eptifibatide and tirofiban can be started 1–2 days before and continued during PCI, whereas abciximab is only recommended during PCI.

Dosing

Abciximab is given as a 0.25 mg/kg bolus followed by a 12-hour infusion of 0.125 μg/kg/min (maximum 10 μg/min). Eptifibatide is given as a 180 μg/kg bolus followed by an infusion of 2 μg/kg/min for 18–24 hours; the dose is reduced to 1 μg/kg/min if the creatinine clearance is less than 50 mL/min. Tirofiban is given as a 0.4 μg/kg bolus followed by an infusion of 0.1 μg/kg/min for 18–24 hours; the dose is reduced by 50% if the creatinine clearance is less than 30 mL/min.

Side-effects

In addition to bleeding, thrombocytopenia is the most serious complication. Thrombocytopenia, which occurs soon after drug administration, is immune-mediated and is caused by antibodies directed against neoantigens on GPIIb/IIIa that are exposed upon antagonist binding. With abciximab, thrombocytopenia occurs in up to 5% of patients. Thrombocytopenia is severe in about 1% of these individuals. Thrombocytopenia is less common with eptifibatide and tirofiban than with abciximab, occurring in about 1% of treated patients. Platelet transfusion is indicated if there is bleeding or if the platelet count falls below 50,000/μL.

Vorapaxar

Mechanism of action

Thrombin is the most potent platelet agonist. Vorapaxar, a synthetic analogue of himbacine, is an orally active inhibitor of type 1 protease-activated receptor (PAR-1), the major thrombin receptor on platelets. Although vorapaxar inhibits platelet PAR-1 in a reversible manner, because of its long half-life (8–12 days) and its high-affinity interaction with PAR-1, vorapaxar functions as an irreversible inhibitor of PAR-1.

Indications

Vorapaxar is indicated for the reduction of thrombotic cardiovascular events in patients with a history of MI or peripheral artery disease and no history of intracranial haemorrhage, stroke or TIA. Vorapaxar is often administered in conjunction with aspirin and/or clopidogrel.

Dosing

Vorapaxar is given orally at a dose of 2.08 mg once daily.

Side-effects

The major side-effect is bleeding, including intracranial and fatal bleeding.

Anticoagulants

There are both parenteral and oral anticoagulants. Currently available parenteral anticoagulants include the heparins (unfractionated heparin [UFH], low-molecular-weight heparin [LMWH] and fondaparinux) and direct thrombin inhibitors (bivalirudin and argatroban). The oral anticoagulants include vitamin K antagonists, such as warfarin, and direct oral anticoagulants (DOACs), which are also termed non–vitamin K antagonist oral anticoagulants (NOACs). The DOACs include dabigatran, which inhibits thrombin, and rivaroxaban, apixaban and edoxaban, which inhibit factor Xa.

Heparins

The heparins include unfractionated heparin (UFH), low-molecular-weight heparin (LMWH) and fondaparinux.

Mechanism of action

UFH is a naturally occurring glycosaminoglycan synthesised by mast cells. Medicinal heparin, which is extracted from porcine intestinal mucosa, consists of polysaccharide chains of alternating uronic acid and glucosamine residues with varying degrees of sulfation. The polysaccharide chains of UFH range in molecular weight from 3000 to 30,000 with a mean molecular weight of 15,000. LMWH is manufactured from UFH by controlled depolymerisation using chemical or enzymatic methods. The LMWH preparations, which include enoxaparin, dalteparin and tinzaparin, have mean molecular weights between 4500 and 5000. Fondaparinux, a synthetic analogue of the antithrombin-binding pentasaccharide, has a

Table 47.2 Comparison of heparin, Lmwh and fondaparinux.

Features	Heparin	LMWH	Fondaparinux
Number of saccharide units	10–30	15–17	5
Catalysis of factor Xa inhibition	Yes	Yes	Yes
Catalysis of thrombin inhibition	Yes	Yes	No
Bioavailability after subcutaneous administration (%)	20	90	100
Plasma half-life (h)	1–4	4	17
Renal excretion	No	Yes	Yes
Induces release of tissue factor pathway inhibitor	Yes	Yes	No
Neutralised by protamine sulphate	Yes	Partially	No

molecular weight of 1728. Table 47.2 compares the features of LMWH and fondaparinux.

UFH and LMWH act as anticoagulants by binding to antithrombin and accelerating the rate at which it inhibits clotting enzymes, particularly thrombin and factor Xa (Figure 47.2). The interaction of UFH and LMWH with antithrombin is mediated by a unique pentasaccharide sequence, which is found on one-third of the chains of UFH and one-fifth of those of LMWH. Fondaparinux is a synthetic analogue of this antithrombin-binding pentasaccharide sequence. Once bound to antithrombin, UFH, LMWH and fondaparinux induce a conformational change in the reactive centre loop that renders it more accessible to its target proteases. This conformational change enhances the rate at which antithrombin inhibits factor Xa by at least two orders of magnitude but has no effect on the rate of thrombin inhibition. To promote thrombin inhibition, UFH and LMWH serve as a template that binds antithrombin and thrombin and brings the enzyme in close apposition to the inhibitor.

Only pentasaccharide-containing heparin chains composed of at least 18 saccharide units (which corresponds to a molecular weight of 5400) are of sufficient length to bridge thrombin and antithrombin together. With a mean molecular weight of 15,000 and a range of 3000–30,000, almost all the chains of UFH are long enough to provide this bridging function. By definition, heparin has an anti-factor Xa to anti-factor IIa (thrombin) ratio of 1:1. In contrast, with mean molecular weights ranging from 4500 to 5000, which correspond to 15–17 saccharide units, at least half of the pentasaccharide-containing chains of LMWH are too short to bridge thrombin to antithrombin. However, these shorter chains retain the capacity to accelerate factor Xa inhibition. Consequently, LMWH catalyses factor Xa inhibition by antithrombin relatively more than thrombin inhibition and has an anti-factor Xa to anti-factor IIa ratio that ranges from 2:1 to 4:1. Because fondaparinux is too short to bridge thrombin to antithrombin, fondaparinux only catalyses factor Xa inhibition.

Heparin and LMWH induce the release of tissue factor pathway inhibitor (TFPI) from the endothelium. Longer heparin chains induce the release of more TFPI than shorter chains; fondaparinux does not induce TFPI release. TFPI binds and inhibits factor Xa and the TFPI-factor Xa complex then inhibits tissue factor–bound factor VIIa, thereby shutting down thrombin generation via the extrinsic pathway of coagulation. Consequently, released TFPI may contribute to the antithrombotic activity of heparin and LMWH.

After entering the circulation, heparin binds to a variety of plasma proteins other than antithrombin, which decreases its anticoagulant activity. The levels of heparin-binding proteins vary between patients because some of these proteins are acute-phase reactants whose levels are elevated in ill patients, whereas others, such as high-molecular-weight multimers of von Willebrand factor and platelet factor 4 (PF4), are released when platelets or endothelial cells are activated by thrombin. The large amounts of PF4 released from platelets in the vicinity of platelet-rich arterial thrombi may locally neutralise the anticoagulant activity of heparin.

UFH is cleared through a combination of a rapid saturable and a much slower first-order mechanism. The saturable phase of UFH clearance reflects binding to endothelial cells and macrophages. Bound UFH is internalised and depolymerised. The slower, non-saturable mechanism of clearance is largely renal. At therapeutic doses, UFH is mostly cleared through the rapid, saturable and dose-dependent mechanism. The complex kinetics of clearance renders the anticoagulant response to UFH nonlinear at therapeutic doses, with both the intensity and duration of effect rising disproportionately with increasing dose. Thus, the apparent biological half-life of UFH increases from 30 minutes after an intravenous bolus of 25 U/kg, to 60 min with a bolus of 100 U/kg, to 150 min with a bolus of 400 U/kg. In contrast to UFH, LMWH and fondaparinux are cleared solely via the kidneys. Consequently, LMWH and fondaparinux are not recommended if the creatinine clearance is less than 30 mL/min.

Indications

UFH, LMWH and fondaparinux are used for thromboprophylaxis in medical and surgical patients, for prevention and treatment of arterial thrombosis, and for initial treatment of VTE. UFH is used for anticoagulation during PCI, haemodialysis, cardiopulmonary bypass and extracorporeal membrane oxygenation (ECMO). LMWH is used less frequently for these indications and fondaparinux is avoided because of the risk of catheter thrombosis or clotting of extracorporeal circuits.

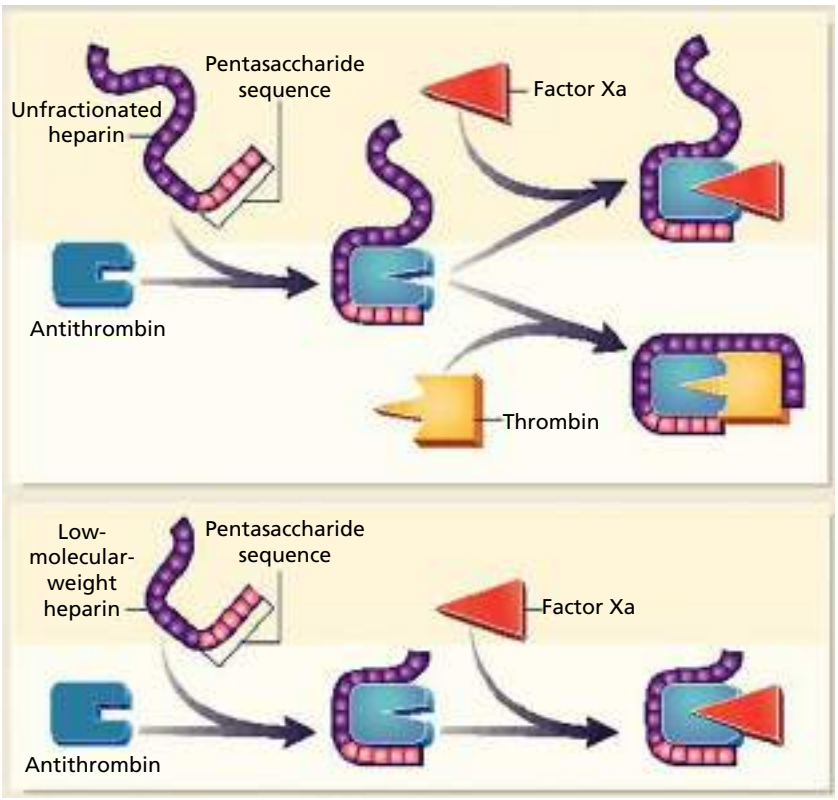

Figure 47.2 Catalysis of antithrombin-mediated inhibition of thrombin or factor Xa by unfractionated heparin (top), low-molecular-height heparin (middle) or fondaparinux (bottom). The interaction of unfractionated heparin and low-molecular-weight heparins with antithrombin is mediated by a unique pentasaccharide sequence. Binding to antithrombin induces a conformational change at the reactive centre loop of antithrombin that accelerates its interaction with factor Xa. Consequently, unfractionated heparin, LMWH and fondaparinux catalyse the inactivation of factor Xa by antithrombin. Catalysis of thrombin inhibition by antithrombin requires the formation of a ternary heparin–antithrombin–thrombin complex, which can be formed only by heparin chains at least 18 saccharide units long. This explains why LMWH has less inhibitory activity against thrombin than unfractionated heparin, and fondaparinux has none.

Dosing

When used for thromboprophylaxis, UFH, LMWH and fondaparinux are given subcutaneously in fixed doses of 5000 units subcutaneously 2 or 3 times daily, 4000–5000 units once daily and 2.5 mg once daily, respectively. For treatment, UFH is given as an intravenous bolus of 5000 units or 70–80 units/kg followed by an infusion of 15–18 units/kg/h. When used for cardiac interventions or for surgery, UFH is often given as a fixed intravenous bolus of 5000–10,000 units or as a weight-adjusted bolus dose of 70–100 units/kg. For treatment, LMWH is given subcutaneously at doses of 150–200 units/kg once daily or 100 units/kg twice daily. Fondaparinux is given subcutaneously at a dose of 7.5 mg once daily; the dose is reduced to 5 mg once daily for those weighing less than 50 kg and increased to 10 mg once daily for those weighing more than 100 kg.

Monitoring

Because the levels of heparin-binding proteins vary between patients, the anticoagulant response to fixed or weight-adjusted doses of UFH is unpredictable. Consequently, coagulation monitoring is essential to ensure that the response is therapeutic when heparin is administered for treatment purposes. The activated partial thromboplastin time (aPTT) is used for monitoring therapeutic doses of UFH, and an aPTT ratio of 1.5–2.5 was initially considered therapeutic because it, corresponded with a heparin level of 0.2–0.4 IU/mL by protamine titration or 0.3–0.7 IU/mL by anti-Xa assay. However, aPTT reagents vary in their responsiveness to UFH so local calibration of aPTT is essential to determine the therapeutic range. Direct monitoring using an anti-Xa assay has advantages over aPTT monitoring, but anti-Xa assays are not standardised and are not available in all hospitals.

LMWH and fondaparinux do not require coagulation monitoring. If monitoring is necessary, anti-factor Xa levels must be measured because LMWH has little effect on the aPTT and fondaparinux has no effect. Therapeutic anti-factor Xa levels with LMWH range from 0.5 to 1.2 U/mL when measured 3–4 hours after drug administration. When LMWH is given in prophylactic doses, peak anti-factor Xa levels of 0.2–0.5 U/mL are desirable.

Side-effects

The most common side-effect of heparins is bleeding. Other complications include thrombocytopenia, osteoporosis and elevated levels of transaminases.

Bleeding

Concomitant administration of antiplatelet or fibrinolytic agents increases the risk of bleeding with heparins, as does recent surgery or trauma.

Thrombocytopenia

A <30% reduction in the platelet count compared with baseline occurs in up to one-third of patients within the first 4 days of starting UFH. This reversible dose-dependent phenomenon is not associated with bleeding or thrombotic complications and does not require cessation of heparin. In contrast, heparin-induced thrombocytopenia (HIT) is a limb and life-threatening process that is triggered by antibodies directed against neoantigens on PF4 that are exposed when heparin binds to this protein (see Chapter 48). These antibodies, which usually are of the IgG subtype, bind simultaneously to the heparin-PF4 complex and to platelet Fc receptors. Such binding activates the platelets and generates platelet microparticles. Circulating microparticles are prothrombotic because they express anionic phospholipids on their surface and can bind clotting factors, thereby promoting thrombin generation.

Typically, HIT occurs 5–14 days after initiation of heparin therapy, but it can manifest earlier if the patient has received heparin within the past 3 months. Even a 50% decrease in the platelet count from the pretreatment value should raise the suspicion of HIT in those receiving UFH (Table 47.3). HIT occurs more frequently with UFH than with LMWH, in surgical patients than in medical patients, and, like many autoimmune disorders, in females than in males. HIT can be associated with venous or arterial thrombosis. Venous thrombosis, which manifests as deep vein thrombosis/or pulmonary embolism, is more common than arterial thrombosis. Arterial thrombosis can manifest as ischaemic stroke or acute MI. Rarely, platelet-rich thrombi in the distal aorta or iliac arteries can cause critical limb ischaemia.

The diagnosis of HIT is established using enzyme-linked assays to detect antibodies against heparin-PF4 complexes or with platelet activation assays. Enzyme-linked assays are sensitive but can be positive in the absence of any clinical evidence of HIT. Because of its sensitivity, a negative enzyme-linked assay result excludes the diagnosis of HIT. The most specific diagnostic test is the serotonin release assay. This test is performed by quantifying serotonin release when washed platelets loaded with labelled serotonin are exposed to patient serum in the absence or presence of varying concentrations of heparin. If patient serum contains HIT antibodies, heparin addition induces platelet activation and subsequent serotonin release.

Heparin should be stopped in patients with suspected or documented HIT, and an alternative anticoagulant should be administered to prevent or treat thrombosis (Table 47.4). The agents most often used for this indication are parenteral direct thrombin inhibitors (such as argatroban or bivalirudin) or parenteral or oral factor Xa inhibitors (such as fondaparinux or rivaroxaban). The alternative anticoagulant should be continued until the platelet count is normal or the diagnosis of HIT is excluded. Warfarin should be avoided in HIT patients because it can trigger skin necrosis and thrombosis. These complications

Table 47.3 Features of heparin-induced thrombocytopenia.

Features	Details
Thrombocytopenia	Platelet count of ≤100,000/μL or a decrease in platelet count of ≥50%
Timing	Platelet count falls 5–10 days after starting heparin
Type of heparin	More common with unfractionated heparin than low-molecular-weight heparin
Type of patient	More common in surgical patients and patients with cancer than general medical patients; more common in women than in men
Thrombosis	Venous thrombosis more common than arterial thrombosis

Table 47.4 Management of heparin-induced thrombocytopenia.

Stop all heparins.
Give an alternative anticoagulant, such as, argatroban, bivalirudin, fondaparinux or rivaroxaban
Do not give platelet transfusions.
Do not give warfarin until the platelet count returns to its baseline level. If warfarin was administered, give vitamin K to restore the INR to normal.
Evaluate for thrombosis, particularly deep vein thrombosis in the lower extremities.

INR, international normalised ratio.

occur because warfarin lowers the levels of protein C and many HIT patients already have low levels of this anticoagulant protein because of increased thrombin generation.

Osteoporosis

Treatment with therapeutic doses of UFH for more than a month can cause a reduction in bone density, which can lead to vertebral fractures. Studies *in vitro* and in mice suggest that this occurs because UFH decreases bone formation and enhances bone resorption. The risk of osteoporosis is lower with LMWH than with UFH; fondaparinux does not appear to affect bone metabolism.

Elevated levels of transaminases

Therapeutic doses of UFH or LMWH can be associated with a modest elevation in serum levels of hepatic transaminases without a concomitant increase in the level of bilirubin. Levels of transaminases rapidly return to normal when the drugs are stopped.

Reversal of heparins

In patients with serious bleeding, the anticoagulant effects of UFH can be completely reversed with protamine sulphate, which is a mixture of basic polypeptides isolated from salmon sperm. Typically, 1 mg of protamine sulphate neutralises 100 U of heparin. Protamine sulphate incompletely neutralises the anticoagulant effects of LMWH because it binds only the longer chains. Because longer chains are responsible for the catalysis of thrombin inhibition by antithrombin, protamine sulphate completely reverses the anti-factor IIa activity of LMWH, but only partially reverses its anti-factor Xa activity and has no effect on the anticoagulant activity of fondaparinux. To avoid anaphylactoid reactions, protamine sulphate is administered by slow intravenous infusion. If available, andexanet alfa reverses the anti-factor Xa activity of LMWH and can be used for fondaparinux reversal.

Parenteral direct thrombin inhibitors

Heparin and LMWH indirectly inhibit thrombin because they require antithrombin to exert their anticoagulant activity. In contrast, direct thrombin inhibitors do not require a plasma cofactor; instead, they bind directly to thrombin and block its interaction with its substrates. Approved parenteral direct thrombin inhibitors include argatroban and bivalirudin. Argatroban is licenced for the treatment of HIT, whereas bivalirudin is approved as an alternative to heparin in patients undergoing percutaneous coronary intervention (PCI), including those with HIT.

Argatroban

Mechanism of action

Argatroban, a univalent inhibitor that targets the active site of thrombin, is metabolised in the liver. Consequently, it must be used with caution in patients with hepatic insufficiency but is not affected by renal impairment. The drug has a half-life of about 45 min.

Indications

Prevention and treatment of thrombosis in patients with HIT.

Dosing

Argatroban is administered by continuous intravenous infusion at a starting dose of 1 mcg/kg/min; the starting dose is decreased to 0.5 mcg/kg/min in patients with hepatic impairment. The aPTT is used to monitor its anticoagulant effect, and the dosage is adjusted to achieve an aPTT 1.5–3 times the baseline value, but not to exceed 100 s. However, responsiveness of aPTT reagents varies and some studies favour thrombin inhibition assays such as the Ecarin chromogenic assay. Argatroban also prolongs the international normalised ratio (INR), a feature that can complicate transitioning of patients from argatroban to warfarin. This problem can be circumvented by using levels of factor X in place of the INR to monitor warfarin. Alternatively, the argatroban infusion can be stopped for 2–3 hours before determination of the INR.

Side-effects

Bleeding is the main side-effect. There is no reversal agent for argatroban.

Bivalirudin

Mechanism of action

A synthetic 20–amino acid analogue of hirudin, bivalirudin is a divalent thrombin inhibitor wherein its amino-terminal portion interacts with the active site of thrombin and its carboxy-terminal tail binds to exosite 1, the substrate-binding domain on thrombin. Bivalirudin has a plasma half-life of 25 min, the shortest half-life of all the parenteral direct thrombin inhibitors. It is degraded by peptidases and partially excreted via the kidneys.

Indications

Bivalirudin is licenced for prevention and treatment of thrombosis in patients with HIT and as an alternative to heparin in patients undergoing PCI or cardiac surgery.

Dosing

In patients with HIT, bivalirudin is administered by continuous intravenous infusion at a dose of 0.15 mg/kg/h if the creatinine clearance is over 60 mL/min; the dose is reduced to 0.08 mg/kg/h

if the creatinine clearance is between 30 and 60 mL/min and to 0.05 mg/kg/h if the creatinine clearance is less than 30 mL/min. When used as an alternative to heparin in patients undergoing PCI, bivalirudin is administered as a bolus of 0.75 mg/kg followed by an infusion of 1.75 mg/kg/h for the duration of the procedure.

Monitoring

When given in high doses for PCI, the anticoagulant activity of bivalirudin is monitored with the activated clotting time. With the lower doses used for HIT, its activity can be monitored using the aPTT. The target aPTT ratio is 1.5–2.5 compared with the baseline value.

Side-effects

Bleeding is the main side-effect. There is no reversal agent for bivalirudin.

Oral anticoagulants

For over 60 years, the vitamin K antagonists, such as warfarin, were the only available oral anticoagulants. This situation changed with the introduction of the DOACs, which include dabigatran, rivaroxaban, apixaban and edoxaban.

Warfarin

A water-soluble vitamin K antagonist initially developed as a rodenticide; warfarin is the most prescribed coumarin derivative.

Mechanism of action

Warfarin interferes with the synthesis of the vitamin K–dependent clotting proteins, which include prothrombin (factor II) and factors VII, IX and X, and anticoagulant proteins, which include protein C, protein S and protein Z.

All the vitamin K–dependent clotting factors possess glutamic acid residues at their amino terminals. A posttranslational modification adds a carboxyl group to the gamma carbon of these residues to generate gamma-carboxy glutamic acid. This modification is essential for expression of the activity of these clotting factors because it enables their calcium-dependent binding to anionic phospholipid surfaces. A vitamin K–dependent carboxylase catalyses the gamma-carboxylation. Thus, vitamin K from the diet undergoes reduction to vitamin K hydroquinone by vitamin K reductase (Figure 47.3). Vitamin K hydroquinone serves as a cofactor for the carboxylase enzyme, which in the presence of carbon dioxide, replaces the hydrogen on the gamma carbon of glutamic acid residues with a carboxyl group. During this process, vitamin K hydroquinone is oxidised to vitamin K epoxide, which then undergoes reduction to vitamin K in a reaction catalysed by vitamin K epoxide reductase.

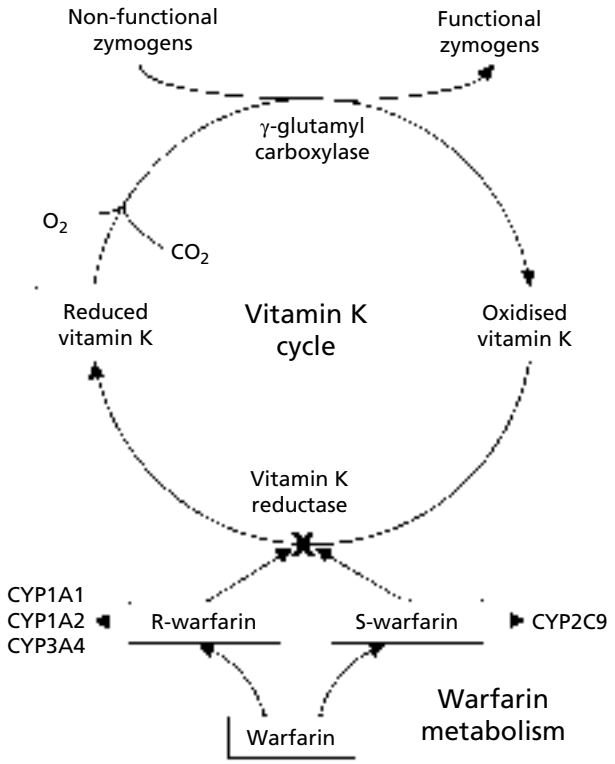

Figure 47.3 Mechanism of action of warfarin. A racemic mixture of *S*- and *R*-enantiomers, *S*-warfarin is the most active. By blocking vitamin K epoxide reductase, warfarin inhibits the conversion of oxidised vitamin K into its reduced form. This inhibits vitamin K–dependent gamma-carboxylation of factors II, VII, IX and X because reduced vitamin K serves as a cofactor for gamma-glutamylcarboxylase, which catalyses the gamma-carboxylation process, thereby converting non-functional zymogens (prozymogens) to functional zymogens capable of binding calcium and interacting with anionic phospholipid surfaces. *S*-warfarin is metabolised by CYP2C9. Common genetic polymorphisms in this enzyme can influence the metabolism of warfarin. Polymorphisms in the C1 subunit of vitamin K reductase (*VKORC1*) can also affect susceptibility of the enzyme to warfarin-induced inhibition, thereby influencing warfarin dosage requirements.

Warfarin inhibits vitamin K epoxide reductase, thereby blocking the gamma-carboxylation process. This results in the synthesis of partially gamma-carboxylated clotting proteins with little or no biological activity. Warfarin exerts its anticoagulant activity when the newly synthesised clotting factors with reduced activity gradually replace their fully active counterparts. The antithrombotic effect of warfarin requires a reduction in the functional levels of factor X and prothrombin, clotting factors with half-lives of 24 and 72 hours, respectively. Because the antithrombotic effect of warfarin is delayed, patients

with established thrombosis or at high risk for thrombosis require concomitant treatment with a rapidly acting parenteral anticoagulant, such as heparin, LMWH or fondaparinux.

Warfarin is a racemic mixture of *R*- and *S*-isomers. It is rapidly and almost completely absorbed from the gastrointestinal tract. Levels of warfarin in blood peak approximately 90 minutes after administration. Racemic warfarin has a plasma half-life of 36–42 hours, and more than 97% of circulating warfarin is bound to albumin. Only a small fraction of unbound warfarin is biologically active.

Warfarin accumulates in the liver, where the two isomers are metabolised via distinct pathways. The more active *S*-enantiomer of warfarin is primarily metabolised by CYP2C9. Two relatively common variants, *CYP2C9*2* and *CYP2C9*3* encode an enzyme with reduced activity. Approximately 25% of Whites have at least one variant allele; these variant alleles are less common in Blacks and Asians. Patients with one variant allele require 20–30% lower maintenance doses of warfarin, whereas those homozygous for these alleles require 50–70% lower doses than do those with the wild-type *CYP2C9*1* alleles. Consistent with the decreased warfarin dose requirement, patients with at least one *CYP2C9* variant allele are at about a twofold higher risk for bleeding compared with individuals with no variant alleles.

Warfarin interferes with the vitamin K cycle by inhibiting the C1 subunit of vitamin K epoxide reductase (VKORCI). Polymorphisms in *VKORC1* can influence the anticoagulant response to warfarin. Several genetic variations of *VKORC1* are in linkage disequilibrium and have been designated as non-A haplotypes. *VKORC1* variants are more prevalent than variants of *CYP2C9*. Asians have the highest prevalence of *VKORC1* variants, followed by Whites and Blacks. The warfarin dose requirements for patients heterozygous or homozygous for the A haplotype are 25 and 50% lower than that needed for patients with the non-A/non-A haplotype. Polymorphisms in *CYP2C9* and *VKORC1* explain up to 25% of the variability in warfarin dose requirements. In addition to genetic factors, fluctuations in the dietary intake of vitamin K, concomitant medications and various disease states influence the anticoagulant effect of warfarin. Consequently, several computerised genotype-based warfarin dosing algorithms also include pertinent patient characteristics, such as age, body weight and concomitant medications. Although these algorithms streamline warfarin dosing, it remains unclear whether their use improves patient outcomes in terms of reducing haemorrhagic complications or recurrent thrombotic events.

Indications

Warfarin is used for the prevention and treatment of venous and arterial thromboembolism.

Dosages

Warfarin is usually started at a dose of 5–10 mg. Lower doses are used for patients with *CYP2C9* or *VKORC1* polymorphisms that affect the pharmacodynamics or pharmacokinetics of warfarin and render patients more sensitive to the drug. The dose is then titrated to achieve the desired target INR. Because of its delayed onset of action, patients with established thrombosis or those at high risk for thrombosis are given concomitant treatment with a rapidly acting parenteral anticoagulant, such as heparin, LMWH or fondaparinux. The initial increase in the INR reflects a reduction in the functional levels of factor VII. Consequently, concomitant treatment with the parenteral anticoagulant should be continued until the INR has been therapeutic for at least 2 consecutive days. A minimum 5-day course of parenteral anticoagulation is recommended to ensure that the levels of prothrombin have fallen into the therapeutic range with warfarin.

Monitoring

Warfarin therapy is most often monitored with the prothrombin time (PT), a test sensitive to reductions in the levels of prothrombin, factor VII and factor X. The test involves the addition of thromboplastin, a reagent that contains tissue factor, phospholipid and calcium, to citrated plasma and determination of the time until clot formation. Thromboplastins vary in their sensitivity to reductions in the levels of vitamin K–dependent clotting factors. Consequently, less sensitive thromboplastins will trigger the administration of higher doses of warfarin to achieve a target PT. This issue can cause problems because higher doses of warfarin increase the risk for bleeding.

The INR was developed to circumvent many of the problems associated with the PT. To calculate the INR, the patient's PT is divided by the mean normal PT, and this ratio is then raised to the power of the international sensitivity index (ISI), an index of the sensitivity of the thromboplastin used for determination of the PT to reductions in levels of the vitamin K–dependent clotting factors. Extremely sensitive thromboplastins have an ISI of 1.0. Most current thromboplastins have ISI values that range from 1.0 to 1.4.

Although the INR has helped standardise anticoagulant practice, problems persist. The precision of INR determination varies depending on reagent-coagulometer combinations, which has led to variability in INR results. Unreliable reporting of the ISI by thromboplastin manufacturers also complicates determination of the INR. Furthermore, every laboratory must establish the mean normal PT with each new batch of thromboplastin reagent. To accomplish this, the PT is measured in newly collected plasma samples from at least 20 healthy volunteers via the same coagulometer that is used for patient samples.

For most indications, warfarin is administered at doses that produce a target INR of 2.0–3.0. An exception is patients with mechanical heart valves in the mitral position or in patients with a mechanical heart valve in other positions who have additional risk factors for stroke, such as atrial fibrillation, in whom a target INR of 2.5–3.5 is recommended. Studies in

patients with atrial fibrillation demonstrate an increased risk for ischaemic stroke when the INR falls below 1.7 and an increase in bleeding with INR values higher than 4.5. These findings highlight the narrow therapeutic window of vitamin K antagonists.

The narrow therapeutic window of warfarin renders frequent monitoring of coagulation necessary to ensure a therapeutic anticoagulant response. Even patients with stable warfarin, dose requirements should have their INR determined every 3–4 weeks. Although some studies have raised the possibility that testing every 12 weeks may suffice in such patients, these results require confirmation in larger numbers of patients. More frequent INR monitoring is necessary with the introduction of new concomitant medications because many drugs enhance or reduce the anticoagulant effects of warfarin.

Side-effects

The major side-effect of warfarin is bleeding; a rare complication is skin necrosis. Uncommon side-effects include vascular calcification, particularly in patients with end-stage kidney disease, alopecia and purple toes. Unless a parenteral anticoagulant is given concomitantly, skin necrosis can occur with the initiation of warfarin therapy in patients with hereditary or acquired deficiency in protein C or protein S because warfarin lowers the levels of these vitamin K–dependent anticoagulant proteins even further prior to exerting its antithrombotic effects. Warfarin crosses the placenta and can cause fetal abnormalities, so it should not be used during pregnancy.

Bleeding

At least half of the bleeding complications with warfarin occur when the INR exceeds the therapeutic range. Bleeding complications may be mild, such as epistaxis or haematuria, or more severe, such as retroperitoneal or gastrointestinal bleeding. Life-threatening intracranial bleeding can also occur. Warfarin-treated patients who experience bleeding when their INR is in the therapeutic range require investigation of the cause of the bleeding.

Skin necrosis

A rare complication of warfarin, skin necrosis usually occurs 2–5 days after initiation of therapy. Well-demarcated erythematous lesions form on the thighs, buttocks, breasts or toes. Typically, the centre of the lesion becomes progressively necrotic. Examination of skin biopsy specimens taken from the borders of these lesions reveals thrombi in the microvasculature.

Warfarin-induced skin necrosis occurs in patients with congenital or acquired deficiencies of protein C or protein S or in patients with HIT whose heparin has been stopped but an alternate anticoagulant has not been given. Initiation of warfarin therapy in these patients produces a precipitous fall in the plasma levels of proteins C and S, thereby eliminating this important anticoagulant pathway before warfarin exerts an antithrombotic effect through lowering of the functional levels of factor X and prothrombin. The resultant procoagulant state triggers thrombosis that is localised to the microvasculature of fatty tissues for unknown reasons.

Treatment of warfarin-induced skin necrosis involves discontinuation of warfarin and reversal with vitamin K if needed. An alternative anticoagulant should be given to patients with thrombosis. Protein C concentrates may accelerate healing of the skin lesions in protein C–deficient patients; fresh frozen plasma may be of value for those with protein S deficiency. Occasionally, skin grafting is necessary in those with extensive skin loss. Because of the potential for skin necrosis, patients with known protein C or protein S deficiency require overlapping treatment with a parenteral anticoagulant when initiating warfarin therapy. Warfarin should be started at low doses in these patients, and the parenteral anticoagulant should be continued until the INR is therapeutic for at least 2–3 consecutive days. The use of rivaroxaban or apixaban in place of warfarin can simplify management of such patients.

Pregnancy

Warfarin crosses the placenta and can cause fetal abnormalities or bleeding. The fetal abnormalities include a characteristic embryopathy, which consists of nasal hypoplasia and stippled epiphyses. The risk for embryopathy is highest with warfarin administration in the first trimester of pregnancy. Central nervous system abnormalities can also occur with exposure to warfarin at any time during pregnancy. Finally, maternal administration of warfarin produces an anticoagulant effect in the fetus that can cause bleeding. This is of particular concern at delivery when trauma to the head during passage through the birth canal can lead to intracranial bleeding. Because of these potential problems, warfarin is contraindicated in pregnancy, particularly in the first and third trimesters. Instead, heparin, LMWH or fondaparinux can be given during pregnancy for prevention or treatment of thrombosis. Warfarin does not pass into breast milk and thus is safe for nursing mothers.

Periprocedural management

There is no need to stop warfarin treatment before procedures associated with a low risk for bleeding such as dental cleaning, simple dental extraction, cataract surgery or skin biopsy. In contrast, warfarin must be stopped 5 days before elective invasive procedures associated with a moderate or high risk for bleeding to allow the INR to return to normal levels. Only patients at high risk for thrombosis while not taking warfarin (such as those with mechanical heart valves in the mitral

position or patients with atrial fibrillation with a prior history of stroke) require bridging with once- or twice-daily subcutaneous injections of LMWH when the INR falls below 2.0. The last dose of LMWH should be given 12–24 hours before the procedure, depending on whether LMWH is administered twice or once daily, respectively. Once haemostasis is secure after the procedure, warfarin can be restarted. Thromboprophylaxis with LMWH can be given starting the day after major surgery and should be continued until the INR is therapeutic

Reversal

In asymptomatic patients whose INR is between 3.5 and 9, warfarin should be withheld until the INR returns to the therapeutic range. If the patient is at high risk for bleeding, sublingual or oral vitamin K at a dose of 1–2.5 mg can be considered. Patients with serious bleeding require 10 mg of vitamin K by slow intravenous infusion with additional doses of vitamin K until the INR is in the normal range together with four-factor prothrombin complex concentrate to replace the missing vitamin K–dependent clotting proteins. Prothrombin complex concentrate is preferred over fresh frozen plasma for warfarin reversal because it normalises the INR more rapidly and because the volume of administration is much smaller.

Direct oral anticoagulants (DOACs)

Mechanism of action

The DOACs include dabigatran, which inhibits thrombin, and apixaban, edoxaban and rivaroxaban, which inhibit factor Xa. These agents, which are small molecules that bind reversibly to the active site of their target enzyme, have a rapid onset of action after oral administration and have half-lives that permit once- or twice-daily administration (Table 47.5). Designed to produce a predictable level of anticoagulation, the DOACs are more convenient than warfarin because they can be given in fixed doses without routine coagulation monitoring.

Indications

All four DOACs are licenced for stroke prevention in patients with non-valvular atrial fibrillation, which encompasses patients without mechanical heart valves or severe rheumatic mitral valve disease, and for treatment of venous thromboembolism (VTE). For these indications, guidelines give preference to DOACs over VKAs such as warfarin for most patients. Exceptions are patients with (i) end-stage kidney disease and a creatinine clearance below 15 mL/min, (ii) triple positive antiphospholipid syndrome (i.e. lupus anticoagulant and elevated levels of anticardiolipin and anti-beta2-glycoprotein I antibodies) and (iii) cancer-associated thrombosis at high risk for bleeding such as those with an unresectable intraluminal gastrointestinal or gastroesophageal tumour or transitional cell cancer of the bladder, and patients with multiple brain metastases.

Dabigatran, rivaroxaban and apixaban are licenced for thromboprophylaxis after elective hip or knee arthroplasty; edoxaban is only licenced for this indication in Japan. Very low dose rivaroxaban is licenced for use with aspirin for secondary prevention in patients with coronary or peripheral artery disease.

Dosing

For prevention of stroke in patients with nonvalvular atrial fibrillation, rivaroxaban is given at a dosage of 20 mg once daily, with a reduction to 15 mg once daily in patients with a creatinine clearance of 15–49 mL/min; dabigatran is given at a dosage of 150 mg twice daily with a reduction to 110 mg twice daily in patients over 80 years of age or over 75 years of age with a risk factor for bleeding such as a creatinine clearance between 30 and 50 mL/min or concomitant use of an antiplatelet agent; the 110 mg dabigatran dosage is not available in the United States where a dosage of 75 mg twice daily is used for patients with a creatinine clearance of 15–30 mL/min and the 150 mg twice daily dose for those with a creatinine clearance over 30 mg/mL; apixaban is given at a dosage of 5 mg twice daily, with a reduction to 2.5 mg twice daily for patients with at least two of the

Table 47.5 Comparison of the pharmacologic properties of the direct oral anticoagulants.

Characteristic	Rivaroxaban	Apixaban	Edoxaban	Dabigatran
Target	Factor Xa	Factor Xa	Factor Xa	Thrombin
Prodrug	No	No	No	Yes
Bioavailability	80%	60%	50%	6%
Dosing	qd (bid)	bid	qd	bid (qd)
Half-life (hours)	7–11	12	9–11	12–17
Renal excretion	33% (66%)	25%	50%	80%
Interactions	CYP3A4/P-gp	CYP3A4/P-gp	P-gp	P-gp

bid, twice a day; CYP, cytochrome P450; P-gp, P-glycoprotein; qd., once a day.

'ABC' criteria (i.e., age over 80 years, body weight under 60 kg and creatinine over 1.5 g/dL); and edoxaban is given at a dosage of 60 mg once daily for patients with a creatinine clearance of 50–95 mL/min and with a reduction to 30 mg once daily for patients with any one of the following criteria: creatinine clearance 15–50 mL/min, a body weight of 60 kg or less, or use of potent P-glycoprotein inhibitors, such as verapamil or quinidine.

For treatment of VTE, dabigatran and edoxaban are started after patients have received at least a 5-day course of treatment with a parenteral anticoagulant such as LMWH; dabigatran is given at a dose of 150 mg twice daily provided the creatinine clearance is over 30 mL/min, and the dosage regimen for edoxaban is identical to that used in patients with atrial fibrillation. In contrast, rivaroxaban and apixaban can be given in all-oral regimens; rivaroxaban is started at a dose of 15 mg twice daily for 21 days and is then reduced to 20 mg once daily thereafter, whereas apixaban is started at a dose of 10 mg twice daily for 7 days and is then reduced to 5 mg twice daily thereafter. For long-term secondary prevention, the dosage of apixaban can be lowered to 2.5 mg twice daily and the dose of rivaroxaban can be lowered to 10 mg once daily, doses that have safety profiles similar to those of placebo and aspirin, respectively.

Dabigatran, rivaroxaban and apixaban are licenced for thromboprophylaxis after elective hip or knee replacement surgery; edoxaban is not licenced for this indication except in Japan. Thromboprophylaxis is started after surgery and is often continued for 30 days in patients undergoing hip replacement and for 10–14 days in patients undergoing knee replacement. In lower-risk patients undergoing hip or knee replacement surgery, a 5-day course of rivaroxaban followed by a 30-day course of aspirin at a dose of 81 mg daily appears to be as effective and safe as extended thromboprophylaxis with rivaroxaban. Dabigatran is given at a dose of 220 mg once daily, whereas rivaroxaban and apixaban are given at doses of 10 mg once daily and 2.5 mg twice daily, respectively.

For secondary prevention in patients with stable coronary or peripheral artery disease, very low dose rivaroxaban (2.5 mg twice daily) is combined with aspirin (81–100 mg once daily) to lower the risk for major adverse cardiovascular or limb events. None of the other DOACs is approved for this indication.

Monitoring

Although administered without routine monitoring, it is helpful to determine the anticoagulant activity of the DOACs in some situations, such as assessment of adherence, detection of accumulation or overdose, identification of bleeding mechanisms and determination of activity before surgery or intervention. For qualitative assessment of anticoagulant activity, the PT can be used for factor Xa inhibitors and the aPTT for dabigatran. However, the PT and aPTT may be normal even in the presence of relevant concentration of drug. Rivaroxaban and edoxaban prolong the PT more than apixaban does. In fact, because apixaban has such a limited effect on the PT, anti-factor Xa assays are needed to assess its activity. The effect of the drugs on tests of coagulation varies depending on the reagents used to perform the tests. Chromogenic anti-factor Xa assays and the diluted thrombin clotting time or ecarin clotting or chromogenic assays with appropriate calibrators provide quantitative assays to measure plasma levels of the factor Xa inhibitors and dabigatran, respectively.

Side-effects

As with any anticoagulant, bleeding is the most common side-effect of the DOACs. Although the risk of intracranial bleeding is lower with the DOACs than with warfarin, the risk for gastrointestinal bleeding is higher with dabigatran (at the 150 mg twice-daily dose), rivaroxaban and edoxaban (at the 60 mg once-daily dose) than with warfarin. Dyspepsia occurs in up to 10% of patients treated with dabigatran; this problem improves with time and can be minimised by taking the drug with food. As small molecules, the DOACs can pass through the placenta. Consequently, these agents are contraindicated in pregnancy, and when used by women of childbearing potential, appropriate contraception is important. Small amounts of rivaroxaban pass into breast milk. Therefore, DOACs should not be used by nursing mothers.

Periprocedural management

Like warfarin, the DOACs must be stopped before surgical procedures associated with a moderate or high risk for bleeding. The drugs should be withheld for 1–2 days or longer if renal function is impaired. After surgery, patients should receive thromboprophylaxis with LMWH until haemostasis is restored, at which point the DOACs can be restarted.

Cardiac procedures such as atrial fibrillation ablation or pacemaker implantation can safely be performed without interruption of the DOACs. However, it may be prudent to hold the dose on the morning of the day of the procedure to avoid intervention at peak drug levels.

Management of bleeding

With minor bleeding, withholding one or two doses of drug is usually sufficient. With more serious bleeding, the approach is like that with warfarin, except that vitamin K administration is of no benefit; the anticoagulant and any antiplatelet drugs should be withheld, the patient should be resuscitated with fluids and blood products as necessary, and the bleeding site should be identified and managed. Coagulation testing will determine the extent of anticoagulation, and renal function should be assessed so that the half-life of the drug can be estimated. Timing of the last dose of anticoagulant is important, and oral activated charcoal may help prevent absorption of drug administered in the past 4 hours, particularly in cases of overdose. If bleeding continues or is life-threatening or if it occurs in a critical organ (e.g. the eye) or in a

closed space (e.g. the pericardium or retroperitoneum), reversal of the anticoagulant should be considered.

Reversal

The pharmacological features of the agents available for DOAC reversal are described in Table 47.6 available for DOAC reversal, and idarucizumab is licenced for dabigatran reversal in patients with serious bleeding or those requiring urgent surgery or intervention. A humanised antibody fragment, idarucizumab binds dabigatran with 350-fold higher affinity than that of dabigatran for thrombin to form an essentially irreversible complex that is cleared by the kidneys. Idarucizumab, which is given intravenously as a 5-g bolus, rapidly reverses the anticoagulant effects of dabigatran and normalises the aPTT, diluted thrombin time or ecarin clot time.

Andexanet alpha is available, depending on local approval, for reversal of rivaroxaban, apixaban and edoxaban. A recombinant variant of factor Xa without catalytic activity, andexanet serves as a decoy to sequester oral factor Xa inhibitors until they are cleared from the circulation. Low or high-dose intravenous andexanet regimens are used. The low-dose regimen starts with a bolus of 400 mg followed by an infusion of 4 mg/min for up to 120 min, whereas the high-dose regimen starts with a bolus of 800 mg followed by an infusion of 8 mg/min for up to 120 min. The low-dose regimen is used for reversal of doses of rivaroxaban or apixaban of 10 mg or 5 mg or less, respectively, or for any dose of rivaroxaban or apixaban if the last dose was taken more than 8 hours prior to presentation. The high dose regimen is used to reverse rivaroxaban or apixaban doses over 10 and 5 mg, respectively, if the last dose was taken less than 8 hours since the presentation, or for reversal if the dose of rivaroxaban or apixaban or the timing of the last dose is unknown. Andexanet alfa is expensive and is not available in all countries and in all hospitals. Because of its cost, andexanet alfa is often reserved for reversal in patients with intracranial bleeds or for bleeds into a closed space such as retroperitoneal or pericardial bleeds. If andexanet is unavailable, the results of prospective cohort studies suggest that four-factor prothrombin complex concentrate (25–50 units/kg) also is effective at restoring haemostasis. If there is continued bleeding, activated prothrombin complex concentrate (50 units/kg) or recombinant factor VIIa (90 μg/kg) can be considered.

Neither andexanet alfa nor prothrombin complex concentrate has been evaluated for reversal in patients requiring urgent surgery or intervention. Furthermore, andexanet alfa not only reverses oral factor Xa inhibitors but also reverses heparin and LMWH. This could be problematic in patients who require cardiac surgery or vascular surgery, procedures where heparin is used routinely. To circumvent this problem, most surgical procedures and interventions can be done without reversal and four-factor prothrombin complex concentrate can be given if necessary. For patients requiring surgery to stop bleeding such as those with a ruptured aortic aneurysm or with bleeding secondary to polytrauma, upfront four-factor prothrombin concentrate administration can be considered.

At an earlier stage of development than andexanet, ciraparantag is a synthetic, cationic small molecule that binds rivaroxaban, apixaban and edoxaban, as well as dabigatran, heparin, LMWH and fondaparinux. When given as an intravenous bolus to volunteers who took rivaroxaban, apixaban or edoxaban, ciraparantag reduced the whole-blood clotting time in a concentration-dependent manner. Additional studies are needed before ciraparantag will be approved.

Table 47.6 Reversal agents for direct oral anticoagulants.

Feature	Idarucizumab	Andexanet Alfa	Ciraparantag
Structure	Humanised antibody fragment	Recombinant human factor Xa variant	Synthetic, small cationic molecule
Mass (Da)	47,776	39,000	573
Mechanism of action	Binds dabigatran with high affinity	Competes with factor Xa for binding	Binds via hydrogen bonds
Target	Dabigatran	Rivaroxaban, apixaban, edoxaban and heparins	Dabigatran, rivaroxaban, apixaban, edoxaban and heparins
Administration	Intravenous bolus	Intravenous bolus followed by a 2-hour infusion	Intravenous bolus
Measurement of reversal	Activated partial thromboplastin time, diluted thrombin time, ecarin clotting time or chromogenic assay	Calibrated anti-factor Xa assays	Whole-blood clotting time
Elimination	Renal (catabolism)	Not reported	Not reported
Cost	Moderate	High	Likely low

Novel anticoagulants in development

Although the DOACs are a major advance over warfarin, the search for more effective and safer anticoagulants continues. Evidence that factor XII and factor XI, components of the contact system, are important for thrombosis but less so for haemostasis has prompted the development of anticoagulants that target these factors. Ongoing studies are evaluating antisense oligonucleotides, inhibitory antibodies and small molecule inhibitors of factor XI.

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CHAPTER 48

Pathogenesis of venous thromboembolism

48

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Introduction

Epidemiology of venous thromboembolism (VTE)

Deep vein thrombosis (DVT) is common, principally affecting the lower limbs. In western populations, the annual incidence is around 1–1.5 per 1000 population. Incidence is age-dependent, being exceptional in childhood and increasing with age with an incidence of around 1 per 10,000 population per annum in young adults, and 1 per 100 per annum in the very elderly. The incidence of pulmonary embolism is lower, at around 1 per 3000 population annually, although subclinical pulmonary embolism occurs frequently in subjects with proximal lower limb DVT. Of patients who die in hospital, there is evidence of pulmonary embolism in up to 20% of cases and in many, it contributes to the cause of death. In some cases pulmonary embolism presents without identifiable DVT, reflecting that 80% of DVT are clinically silent. Venous thrombosis can of course develop in any part of the circulation and indeed in certain conditions, there is a predilection for specific sites such as in the splanchnic circulation in patients with myeloproliferative neoplasms or in the cerebral veins in patients with the rare vaccine induced thrombocytopenia and thrombosis syndrome (VITT).

Virchow's triad, described in 1859, postulates that venous thrombosis may result from changes in blood flow, hypercoagulability of the blood or abnormalities of the blood vessel wall. Each of these features could arise from genetic or acquired conditions and acquired conditions may be persistent or transient. Venous thrombosis should be viewed as a multicausal disease in which genetic and acquired risk factors interact synergistically. This effect may be additive or multiplicative. The interaction between combined oral contraceptive preparations and factor V Leiden is probably the best-documented description of this synergistic relationship between inherited and acquired risk factors (Rosendaal Diagram as Figure 48.1). Understanding gene–environment interactions and the concept of multiple coinciding risk factors is necessary to recognise periods of increased thrombosis risk for patients. Modern management of proven episodes of VTE emphasises the detection, or not, of risk factors that might determine the duration of anticoagulation and the use of validated scoring systems to predict the risk of recurrence of thrombosis following withdrawal of anticoagulant therapy in those sustaining otherwise 'unprovoked' episodes of VTE. Conditions deemed to be risk factors for VTE are shown in Table 48.1.

Hereditary thrombophilia

Familial thrombophilia is likely a multiple-gene disorder. Family members of 'thrombosis-prone families' may have one or more defects some of which will be identifiable in the laboratory whilst others are not.

In most instances, heritable thrombophilia is a late-onset genetic disease with low penetrance. There is no internationally accepted definition of thrombophilia. A clinical definition of heritable thrombophilia describes an inherited tendency for venous thrombosis.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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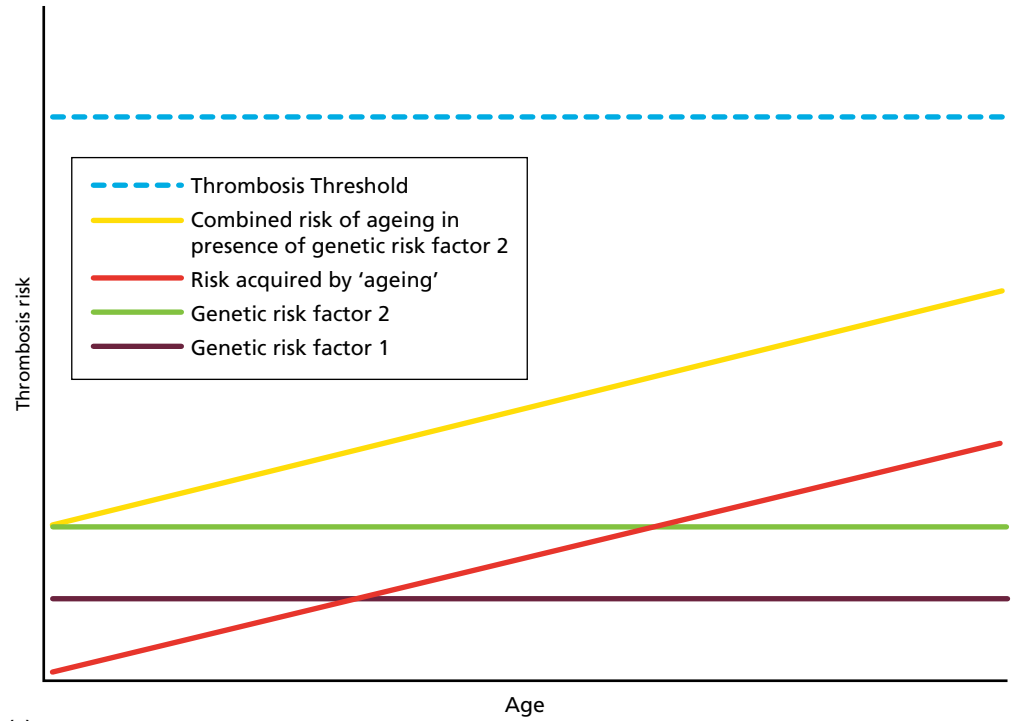

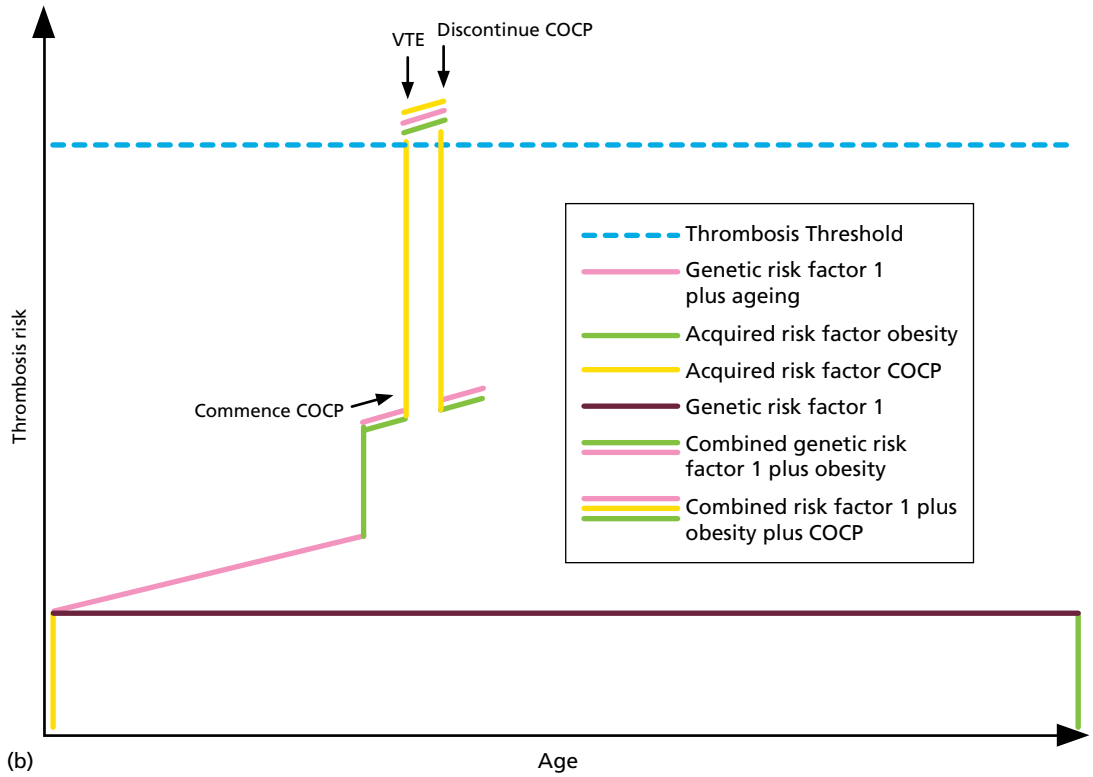

Figure 48.1 (a) The model assumes that there is a thrombosis threshold that, when breached may result in the development of VTE. Numerous risk factors, inherited and acquired, permanent and temporary can contribute to the risk of developing thrombosis. It is well recognised that thrombosis risk increases with age probably due to the acquisition of factors that result in vascular degeneration and hypercoagulability. Thrombosis-prone families, such as those with factor V Leiden have an inherited risk of thrombosis which is constant in

Deficiencies of the natural anticoagulants antithrombin, protein C and protein S have been linked with familial thrombosis. The causal association between these heritable thrombophilias and venous thrombosis has been confirmed by comparing the prevalence of defects in patients with venous thrombosis and controls. In recent years, several other potential thrombophilic risk factors have been investigated but only the *F5*G1691A (FVR506Q, FV Leiden) and the *F2*G20210A gain of function gene mutations have been shown to be unequivocally associated with an increased risk of thrombosis, that is an odds ratio (OR) of ≥2. The prevalence of the heritable thrombophilias varies across populations and the relative risk (RR) for thrombosis development (the probability of the disease in those with the risk factor divided by the probability of the disease in those without the risk factor) or the odds ratio (OR) (the odds of the disease in those with the risk factor divided by the odds of the disease in those without the risk factor) ranges from around 2, for the *F2*G20210A, to 20 for antithrombin deficiency.

It was hoped that the availability of laboratory tests for these conditions would significantly help to inform clinical decision-making within families and also in patients with a first episode of unprovoked VTE. It is now apparent that testing for heritable thrombophilia typically does not predict the likelihood of recurrence in the majority of unselected patients with symptomatic venous thrombosis (Figure 48.2). Furthermore, testing for inherited thrombophilia has not been shown to reduce recurrence of venous thrombosis in clinical studies. There is a low risk of thrombosis in affected asymptomatic relatives of patients with thromobophilic defects followed prospectively and the results of thrombophilia tests are, as such, frequently given undue weight resulting in dubious clinical decision-making with regard to the management of periods of risk and exposure to risk factors. Given these considerations, the clinician must consider the potential clinical utility of testing for heritable thrombophilic defects in each individual patient or family affected by venous thrombosis.

Heritable thrombophilia is subject to a strong gene–environment interaction. For example, there is a synergistic increase in the risk of venous thrombosis due to an interaction between the factor V Leiden mutation and oestrogen exposure and obesity. Similar observations have been made in antithrombin-deficient families where venous thrombosis frequently occurs when an affected individual is exposed to an environmental risk. This was evident in a study of families with type 1 antithrombin deficiency, where the incidence of venous thrombosis was 20 times greater in affected family members, but was strongly dependent on the presence of concurrent acquired risk factors. In this study, the annual incidence of venous thrombosis in affected family members in any year in which they were exposed to surgery, trauma, plaster cast immobilisation or hospitalisation was 20% (provoked venous thrombosis). However, in any year in which there was no exposure the incidence of VTE was only 0.3% (i.e. unprovoked venous thrombosis rate). This is very much in keeping with the multicausal model for VTE described by Rosendaal shown in Figure 48.1.

Antithrombin deficiency

The gene for antithrombin (*SERPINC1* and previously termed antithrombin III) is located on the long arm of chromosome 1 and contains seven exons spanning 13.5 kb encoding a glycoprotein of 464 amino acids, 58 kDa and plasma concentration 2–3 μmol/L. Antithrombin is a serpin (serine protease inhibitor) and its primary targets are thrombin, factor Xa and factor IXa. Its molar concentration in the circulation is higher than that of prothrombin, and as only a fraction of prothrombin is converted to thrombin during coagulation, antithrombin is present in vast excess over the protease, although the site will determine the local concentrations of the enzyme and inhibitor. The plasma half-life of antithrombin is about 90 hours.

The serpin mechanism was confirmed following resolution of a crystal structure of the final serpin–protease complex in 2000. In contrast to the classical lock-and-key mechanism of protease inhibition, serpins form a stable covalent complex in which there is a

level over time (genetic risk factor 1). Some genetic factors are associated with a higher risk than others (genetic risk factor 2). The combined effect of genetic risk factor 2 and increasing age is shown). The model shows the increasing risk of thrombosis with age and also the increased thrombosis risk associated with the combined effects of an inherited and acquired risk factors. (b) This figure demonstrates the effect on thrombosis of combinations of risk factors. The figure assumes a thrombosis threshold and that thrombosis risk increases with age as outlined in (a). The level of risk of thrombosis associated with genetic risk factor 1, use of the combined oral contraceptive and with obesity are all similar as shown in the figure. The figure demonstrates the effect of the concurrence of risk factors in the development of VTE. In some situations the concurrence of risk factors, such as genetic risk factor 1 and obesity results in an additive effect while the concurrence of genetic risk factor 1 and the COCP has a multiplicative effect on thrombosis risk. The temporary and reversible nature of thrombosis risk factors, which may result in recommendations of finite periods of anticoagulation are also demonstrated. In the figure, the effect of ageing combined with genetic risk factor 1 is shown. The patients thrombosis risk increases as obesity develops. The addition of the COCP to genetic risk factor 1 combined with obesity results in clinical VTE. When the patient develops VTE, the COCP is stopped and the risk of thrombosis returns to that associated with the combination of genetic risk factor 1 and obesity.

Table 48.1 Risk factors for venous thromboembolism.

Familial thrombophilia
- Antithrombin deficiency
- Protein C deficiency
- Protein S deficiency
- FVR506Q (Factor V Leiden)
- F2G20210A (Prothrombin mutation)

Inevitable/environmental
- Increasing age
- Pregnancy and puerperium
- Prolonged immobility, e.g. long-haul travel
- Obesity
- Trauma

Iatrogenic
- Surgery
- Indwelling venous devices
- Pharmacological
- Oestrogen-related: combined oral contraceptive, hormone-replacement therapy, tamoxifen
- Ovarian stimulation regimens
- Chemotherapy and radiotherapy
- Heparins (immune reaction)

Disease-related
- Antiphospholipid syndrome
- Cancer
- Myeloproliferative neoplasms
- Acute promyelocytic leukaemia
- Chronic inflammatory states e.g. nephrotic syndrome, Behcet's syndrome, inflammatory bowel disease, connective tissue disorders
- Acute inflammatory disorders e.g. pneumonia, sepsis.
- Stroke
- Paroxysmal nocturnal haemoglobinuria
- Thrombotic thrombocytopenic purpura
- Sickle cell disease and other sickling syndromes
- Congestive cardiac failure

dramatic conformational change in both the inhibitor and the inhibited protein that alters the properties of each, resulting in rapid clearance from the circulation. Antithrombin consists of three β-sheets and nine α-helices organised into an upper β-barrel domain and a lower helical domain. These two domains are bridged by the main structural feature of a serpin, the central A sheet. The flexible reactive centre loop (RCL) contains the scissile bond formed by the P1 arginine and P1′ serine. Amino acids in the RCL are designated P1, P2 and so forth, moving towards the N-terminus of the peptide sequence and P1′, P2′ and so forth moving towards the C-terminus of the sequence. The length and flexibility of the loop render it highly susceptible to proteolysis. Cleavage of the RCL by thrombin, FXa or FIXa produces a conformational change during which the RCL inserts as the central strand of the expanded central A sheet. This results in translocation of the protease to the opposite pole of the serpin. The incorporation of the RCL as the central strand produces a hyperstable molecule such that the protease is crushed by intermolecular clashes with the hyperstable serpin. Distortion of the active site architecture disrupts the catalytic triad and results in disorganisation of almost 40% of the protease architecture. The poor inhibitory activity of the circulating native form of antithrombin is conferred by intramolecular contacts that restrain the RCL and orientate the P1 arginine away from attacking proteases. Activation via release of the RCL is favoured by binding of glycosaminoglycans to the 'heparin-binding site' located in the region of the D-helix. An initial low-affinity interaction induces high-affinity binding (referred to as an induced fit mechanism) and this produces a conformational change in the antithrombin that releases the RCL for protease attack. Activation increases the rate of protease inactivation 1000-fold. Successful inhibition of the protease depends on a race between complete proteolysis of the RCL with release of the regenerated protease from the cleaved loop (the substrate pathway) and incorporation of the cleaved loop into the central A-sheet with resultant capture and crushing of the tethered protease (the inhibitory pathway). Some mutations in the *SERPINC1* gene produce a dysfunctional antithrombin molecule in which the insertion of the RCL into the central A-sheet is retarded, turning the molecule into a substrate rather than an inhibitor. This is an example of how this highly regulated mechanism of thrombin or FXa inhibition renders antithrombin susceptible to dysfunction through genetic mutation.

Two laboratory phenotypes of heritable antithrombin deficiency are recognised. Type 1 is characterised by a quantitative reduction of antithrombin, with a parallel reduction in function and the level of protein in the plasma measured immunologically as the antigenic level. Type 2 deficiency is due to the production of a qualitatively abnormal antithrombin protein. The functional activity is discrepantly low compared with the antigenic level. Type 2 deficiency is subclassified according to the nature of the functional defect:

- Type 2 reactive site (*RS*): mutations alter the sequence of the RCL, reducing the ability to inhibit thrombin or FXa in the presence or absence of heparin in a laboratory assay.
- Type 2 heparin-binding site (*HBS*): mutations affect the ability of antithrombin to bind to and be activated by glycosaminoglycans and therefore result in reduced ability to inhibit thrombin or FXa only in the presence of heparin in a laboratory assay. These mutations are typically located in the designated 'heparin-binding site' in the region of the D helix of the molecule.
- Type 2 pleiotropic (*PE*): a single mutation produces multiple effects on the structure–function relationship of the molecule, particularly the interaction between heparin binding and RCL, often associated with low plasma levels due to either effects on secretion or instability.

Approximately 100-point mutations (missense, nonsense or insertions or deletions causing frameshifts) and several whole

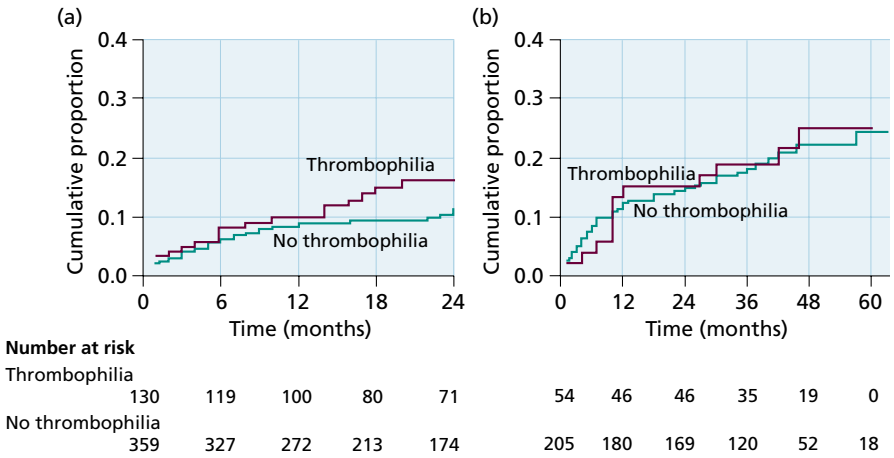

Figure 48.2 Cumulative recurrence of venous thrombosis in consecutive patients with and without heritable thrombophilia followed from the end of anticoagulation after a first episode of venous thrombosis in the first (a) and second (b) Cambridge cohort studies. The hazard ratio for recurrence in patients with thrombophilia compared with those without was 1.3 (95% CI 0.8–2.0) and 1.0 (95% CI 0.5–2.1), respectively. In the second cohort, patients with postoperative thrombosis were excluded, which may account for the slightly higher recurrence rate. (Source: For related publications on these cohorts, see Baglin *et al.* (2003) and Baglin T, Palmer CR, Luddington R, Baglin C (2008) Unprovoked recurrent venous thrombosis: prediction by D-dimer and clinical risk factors. *J. Thromb. Haemost.* **6:** 577–82.)

or partial gene deletions have been identified as causes of type 1 deficiency. Numerous point mutations causing qualitative type 2 deficiency have been identified. Homozygous type 1 deficiency and probably homozygous type 2 *RS* mutations are incompatible with life. Homozygous type 2 *HBS* and *PE* mutations are compatible with life but are associated with increased risk of thrombosis and adverse pregnancy outcome.

Protein C deficiency

The gene for protein C (*PROC*) is located on chromosome 2 and contains nine exons spanning 11 kb. Protein C is a vitamin-K-dependent glycoprotein synthesised in the liver (419 amino acids, 62 kDa, plasma concentration 65 nmol/L). Its concentration in plasma is about 100 times less than that of antithrombin. The plasma half-life of protein C is about 6 hours.

Protein C is the zymogen precursor of activated protein C (APC). Protein C is activated by thrombin bound to thrombomodulin on the endothelial surface. This is facilitated by the endothelial protein C receptor (EPCR). APC acts as a physiological anticoagulant by cleaving the cofactors FVa and FVIIIa so reducing thrombin generation. Protein S is a cofactor for APC-mediated inactivation of FVa and FVIIIa. APC has an anti-inflammatory effect on endothelium via cleavage of PAR1 and also reduces pro-inflammatory cytokines such as IL-6, IL-8 and TNFα and upregulates anti-inflammatory cytokines such as IL-10. These actions may also be relevant to thrombosis risk.

Protein C deficiency is classified into type 1 and 2 defects on the basis of functional and antigenic assays. The relative risk of thrombosis in relation to type 1 and the various type 2 defects has not been characterised. Most heritable protein C deficiency is due to type 1 deficiency. The majority of type 1 defects are due to point mutations. Multiple type 2 defects have been reported involving the catalytic active site, the phospholipid-binding Gla domain, the propeptide cleavage activation site and sites of interaction with substrates or cofactors.

Protein S deficiency

The gene for protein S (*PROS*) is located on chromosome 3 and contains 15 exons spanning 80 kb. A pseudogene with approximately 95% homology is located adjacent to *PROS* and this can cause difficulty in the identification of mutations within *PROS*.

Protein S is a vitamin-K-dependent glycoprotein produced in the liver, endothelial cells and megakaryocytes (676 amino acids, 69 kDa, plasma concentration of free protein 145 nmol/L). Protein S is a non-enzymatic cofactor for APC-mediated inactivation of FVa and FVIIIa. Approximately 60% circulates bound to C4b-binding protein and this portion is inactive. The remaining 40%, designated free protein S, is uncomplexed and is the active form. Free protein S increases the affinity of APC for negatively charged phospholipid surfaces on platelets or the endothelium and increases complex formation of APC with the activated forms of FV (FVa) and FVIII (FVIIIa). The binding of protein S by C4b-binding protein is very strong and free protein S is essentially the molar excess of protein S over C4b-binding protein. In addition to APC cofactor activity, protein S has an independent anticoagulant effect by acting as a cofactor for TFPI (tissue factor pathway inhibitor that inhibits FVIIa and FXa).

Protein S defects have been divided into three types. In type I deficiency, both total and free protein S antigen levels are low (and functional activity is low if measured). A type III deficiency

characterised by a normal level of total protein S, but a reduced level of the free protein S has been described. This may be a phenotypic variation resulting from the same genetic mutations associated with type I deficiency (not recognised with the less sensitive assay for total protein S), but many patients with an apparent type III phenotype do not have heritable protein S deficiency and this may be related to the increase in C4b-binding protein levels with age. Type II defects are characterised by reduced activity in the presence of normal total and free levels of protein S. Type II deficiency is difficult to diagnose because functional protein S assays are imprecise.

FVR506Q (FV)

The gene for FV is located on chromosome 1. It contains 25 exons spanning 80 kb. FV (2196 amino acids, 330 kDa, plasma concentration of free protein 30 nmol/L) is the cofactor for activation of prothrombin by FXa in the prothrombinase complex. The plasma half-life is 15 hours. Factor V has no cofactor activity until cleaved by thrombin or FXa at Arg709, Arg1018 and Arg1545. In 1993, the phenomenon of resistance to APC in patients with venous thrombosis was described. Patient plasma was resistant to the anticoagulant effect of added exogenous APC. The effect was measured by dividing the activated partial thromboplastin time (APTT) in the presence of added APC by the APTT in the absence of added APC. APC resistance was defined as a ratio less than 2.0. Increased APC resistance was shown to cosegregate with thrombosis in families with familial venous thromboembolism and it was reported that the majority of patients with familial APC resistance have a point mutation, guanine to adenine transition at nucleotide 1691, in exon 10 of the *F5* gene. This encodes what is known as the FV Leiden variant (FVR506Q). FV Leiden has normal procoagulant activity, but slower inactivation by APC. Prothrombinase activity is dependent on FVa maintaining the integrity of the enzymatic complex on a phospholipid surface. Thus thrombin generation is limited by FVa, and the normal inactivation of FVa by APC attenuates thrombin generation. Inactivation of FVa occurs through cleavage of the proteins at positions Arg306, Arg506 and Arg679 by APC. Initial cleavage at R506 is necessary for subsequent cleavage at R306 and R679. Mutation of these residues (*F5*G1691A, *FV*R506Q or FV Leiden; *F5*G1091C, *FV*A306T or FV Cambridge) results in resistance to inactivation by APC. The *FV*R506Q variant is present in about 5% of Caucasians with highest rates in white Northern Europeans. The mutation is far less frequent in Africans, Asians and races of Asian ancestry. The high prevalence suggests positive selection but could also be a founder effect.

APC resistance in the absence of *FV*R506Q is found in about 5% of patients with a history of venous thrombosis. This is often related to high FVIII levels or low FV levels, which may be found in association with the *F5*HR2 haplotype. There is evidence that APC resistance in the absence of *FV*R506Q is also associated with venous thrombosis, but defining the cause of resistance in individual patients has no clinical utility. Acquired APC resistance is commonly observed in pregnancy and combined oral contraceptive users.

*F2*G20210A

The gene for prothrombin is located on chromosome 2 and contains 14 exons spanning 21 kb. Prothrombin is a vitamin-K-dependent protein synthesised in the liver (579 amino acids, 72 kDa, plasma concentration 2 μmol/L). Prothrombin is the zymogen precursor of thrombin.

A single nucleotide change, guanine to adenine at position 20210 in the 3′ untranslated region of the prothrombin gene (*F2*G20210A), is associated with elevated plasma prothrombin levels and an increased risk of venous thrombosis. The prevalence of the *F2*G20210A variant is about 2% of whites, with a higher prevalence in southern compared with northern Europeans. The mutation increases the plasma level of prothrombin by about 30%, but the mechanism has not been explained. No specific clotting test for the presence of the mutation has been described and diagnosis depends on DNA analysis.

Other natural anticoagulants

Tissue factor pathway inhibitor (TFPI) is an inhibitor of FXa and tissue factor-bound FVIIa. TFPI knockout is embryonically lethal in mice. TFPI is predominantly on the vascular wall and so measurement in plasma samples is not representative of total TFPI availability. In a few studies, plasma TFPI levels were slightly lower in patients with venous thrombosis than in normal individuals but it is not clear that low levels are a risk factor for venous thrombosis. Protein Z is a vitamin-K-dependent protein that circulates in complex with PZI (protein-Z-dependent protease inhibitor) and catalyses the inhibition of FXa. A causal relationship between PZI and/or PZ deficiency and venous thrombosis has not been demonstrated. Heparin cofactor II, a serpin with a similar structure–function relationship to antithrombin, inhibits thrombin when activated by glycosaminoglycans. Heparin cofactor II knockout mice are healthy and do not develop spontaneous thrombosis, but do have a shorter time to thrombosis in an arterial injury model. A relationship between heparin cofactor II deficiency and thrombosis in humans is uncertain. A relationship between thrombosis and mutations affecting thrombomodulin or EPCR has not been established.

Other procoagulant factors

Increased levels of factors VIII, IX and XI have been associated with an increased risk of VTE. The observed association between elevated VIII levels and VTE are possibly accounted for by the effect of ABO blood group on von Willebrand factor

levels. Recent GWAS however, describe an association between thrombosis risk and rs11420917, which is located upstream from the factor VIII gene on the X chromosome. GWAS studies also identify associations between VTE and loci near the factor 5, 2, 11 and *FGA::FGG* genes. Several genes that do not code for coagulation factors have recently been described in association with thrombosis on chromosome 19 with SLC44A2, a choline transmembrane transporter, ILF3, an interleukin enhancer binding factor, and AP1M2, a subunit of the heterotetrameric adaptor related protein complex, the most plausible candidate genes.

There is equivocal evidence for a causal relationship between fibrinogen levels and venous thrombosis. Elevated fibrinogen levels per se are associated with thrombosis and *FGA::FGG* does show a significant signal in GWAS studies. In addition, approximately 300 examples of heritable dysfibrinogenaemia have been reported. These are suspected when there is a significant discrepancy between the functional Clauss assay and a clot weight or antigen assay. Further investigation is by genetic analysis. The majority of dysfibrinogenaemias are asymptomatic or associated with increased bleeding and very few examples are considered to be clearly associated with an increased tendency to thrombosis, which may be arterial, venous or a combination. Dysfibrinogenaemia has been found in less than 1% of patients with a history of venous thromboembolism and under 10% of patients with dysfibrinogenaemia will sustain a thrombosis. It is associated with postpartum thrombosis and an increased risk of pregnancy loss. The identification and characterisation of rare dysfibrinogenaemias associated with thrombosis is beyond the capability of most coagulation laboratories. Polymorphisms in the prothrombin gene that may further increase the risk of venous thrombosis associated with the *F2*G20210A mutation have been described but their effect is mild. A polymorphism in the *FXIII* gene (*FXIIIV341L*) is significantly less common in patients with coronary heart disease than in control subjects, and a protective effect for venous thromboembolism has been reported.

Fibrinolysis

A causal relationship between levels of individual specific proteins involved in regulating fibrinolysis has not been established. However, a global measure of fibrinolytic potential as measured by a plasma-clot lysis assay was associated with an approximately twofold increased risk of venous thrombosis in patients with clot lysis times above the 90th percentile of controls in the Leiden Thrombophilia Study. Analysis of the MEGA (Multiple Environmental and Genetic Assessment) study confirmed this finding and demonstrated that hypofibrinolysis in combination with established acquired and genetic risk factors, such as *FV*R506Q, had a synergistic effect on venous thrombosis risk. A genetic basis for hypofibrinolysis in these patients was not investigated. Genetic mutation in the thrombin-activatable fibrinolytic inhibitor (*TAFI*) gene has not been linked to thrombosis risk, although plasma TAFI levels appeared to be weakly linked to thrombosis risk in some studies.

Treatment of patients with venous thrombosis and heritable thrombophilia

There is no evidence that the presence of heritable thrombophilia should influence the intensity of anticoagulation or thromboprophylaxis. In patients with antithrombin deficiency, heparin resistance is infrequent and recurrence or extension of thrombosis while on treatment is no greater than that observed in individuals without antithrombin deficiency. Warfarin-induced skin necrosis is extremely rare, even in patients with protein C or S deficiency. The intensity of maintenance therapy with warfarin should not be influenced by laboratory evidence of inherited thrombophilia. There is no evidence that recurrence on treatment is more likely in patients with heritable thrombophilia.

Long-term prospective cohort outcome studies have shown that finding a heritable thrombophilia does not usefully predict recurrence in unselected patients after an episode of venous thrombosis. However, these studies were not powered to exclude an increased risk of recurrence in relation to the rare thrombophilias, such as antithrombin or protein C deficiency. Therefore, it remains uncertain if mutations in *SERPINC1*, *PROC* or *PROS* causing deficiency of the corresponding protein sufficiently predict a high enough risk of thrombosis to justify lifelong anticoagulation. Studies in thrombosis-prone families indicate a risk of recurrence that would justify long-term anticoagulation, but these studies may not be representative of risk associated with the *SERPINC1*, *PROC* and *PROS* mutations per se, but may be influenced by additional defects in the thrombosis-prone family members. Further studies are required to define the relative risk of recurrent events associated with the actual *SERPINC1*, *PROC* and *PROS* mutations.

Systematic reviews indicate a relative risk of 1.4 for recurrent venous thromboembolism in patients heterozygous for the *FV*R506Q mutation and 1.2–1.7 for the *F2*G20210A mutation compared to other patients with idiopathic VTE. The authors concluded that the magnitude of the increase in risk was modest and by itself did not justify an extended duration of anticoagulation. An analysis of the MEGA study showed that testing for inherited thrombophilia did not reduce recurrence of venous thrombosis.

In addition, three key studies, to guide clinical practice, have attempted to determine the risk factors and rates of recurrence of thrombosis following a first unprovoked event. In the DASH study, HERDOO2 and the Vienna prediction model for Thrombosis recurrence, routine testing for familial thrombophilia and antiphospholipid syndrome was not carried out but patients known to have 'strong thrombophilia' were excluded.

Based on the size of these studies and the rarity of 'strong thrombophilia', it was felt unlikely that routine testing would have been of value. Based on these and the results of the Cambridge study above (Figure 48.2), the following conclusions seem reasonable:

- Indiscriminate testing for heritable thrombophilias in unselected patients presenting with a first episode of venous thrombosis is not indicated.
- Decisions regarding duration of anticoagulation (lifelong or not) in unselected patients should be made based on whether an episode of venous thrombosis is provoked or unprovoked, independent of thrombophilia status. The use of the predictive scoring systems above should be considered in all patients with unprovoked events.
- Testing for heritable thrombophilias in selected patients, such as those with a strong family history of unprovoked recurrent thrombosis, may influence decisions regarding duration of anticoagulation and the information given to the patient. Unfortunately, in this regard, identifying patients for testing is not straightforward, as criteria for defining thrombosis-prone families have not been validated and the association between family history of thrombosis and detection of inherited thrombophilia is weak.

Case finding in heritable thrombophilia and thrombosis-prone families

Testing for heritable thrombophilias in patients presenting with venous thrombosis theoretically allows case finding of affected asymptomatic family members. The rationale for this is that it permits avoidance of future environmental risks (e.g. use of the combined oral contraceptive pill) or provides an opportunity for targeted thromboprophylaxis at times of high risk (such as surgery). This rationale assumes, however, that such action would not be taken for family members tested and found not to have the thrombophilic abnormality found in their kin. It also does not account for a situation where a young individual family member who suffers from an unprovoked event at a young age has negative thrombophilia tests. Individual risk is affected by multiple genetic and environmental factors that are different, even amongst first-degree relatives. Prospective cohort studies have determined the annual risk of venous thrombosis in asymptomatic family members identified after testing unselected patients presenting with venous thrombosis. The annual risk of venous thrombosis in asymptomatic family relatives of index patients was 0.1% for those with *F2*G20210A, 0.6% for those with *FV*R506Q, about 2% for protein C and protein S deficiency, and 4% for antithrombin deficiency. Identifiable high-risk periods contributed to approximately half of all events in patients with *FV*R506Q and thromboprophylaxis appears to reduce risk. However, it is the case that during many high-risk periods individuals with a family history of thrombosis are routinely offered thromboprophylaxis independent of thrombophilia test results.

Given the uncertainty, some experts argue that it is reasonable to perform testing if it is anticipated that clinical management will be influenced, for example an intensified or extended period of prophylaxis during a high-risk period. If a family history suggests a high degree of genetic penetrance, then it might be reasonable to test an asymptomatic patient and then their relatives with a view to enhanced prophylaxis at times of high risk in affected members. In all cases, the risks, benefits and limitations of testing should be discussed in the context of explained inheritance and disease risk. The importance of this is demonstrated by reported anxiety after testing positive and an overestimated perception of risk in tested individuals. Simple methods for quantifying a positive family history do not discriminate patients with and without thrombophilia and therefore the decision to test for inherited thrombophilia cannot be accurately guided by the presence or absence of a family history of thrombosis.

Purpura fulminans

Purpura fulminans is a rare syndrome characterised by progressive haemorrhagic skin necrosis that occurs in neonates with congenital severe protein C or S deficiency at birth or in the first few days of life, and in association with infection in children and adults. The condition may occur in children without congenital anticoagulant deficiency following viral infections, with an onset within 10 days of infection. Acquired severe protein S deficiency has been reported in purpura fulminans following chickenpox infection. With bacterial infections, disseminated intravascular coagulation (DIC) is often present, for example meningococcal infection. In patients with very severe skin necrosis, testing for acquired protein C or S deficiency should be considered, as plasma exchange may be beneficial. Neonates homozygous for protein C or S deficiency may be born with skin necrosis and extensive thrombosis.

Vitamin K antagonist-induced skin necrosis

Vitamin K antagonist-induced skin necrosis, such as that seen with warfarin or other coumarins, is extremely rare, even in patients with protein C or S deficiency, such that most individuals with protein C or S deficiency do not develop skin necrosis. However, a high proportion of patients with vitamin K antagonist-induced necrosis have heritable protein C deficiency. The condition has been described in patients with other heritable thrombophilias. Vitamin K antagonist-induced necrosis associated with heritable thrombophilias tends to involve a central distribution especially targeting the buttocks and thighs.

Neonatal stroke (see also Chapter 50)

Heritable thrombophilic defects are associated with perinatal brain injury. The pathogenesis is thrombosis in either cerebral arteries or cerebral veins or sinuses. Thrombophilia is not a significant risk factor for ischaemic stroke in adults, as it does

not increase the risk of arterial thrombosis. Cerebral vein thrombosis can present clinically as stroke and this should be borne in mind but is usually obvious on imaging of the patient. In the neonate, the pathophysiology of stroke is quite different and thrombophilia may increase the risk of arterial thrombosis and stroke in the neonate or fetus.

Laboratory methods and testing strategy for heritable thrombophilia

The laboratory diagnosis of heritable thrombophilia is difficult as the tests are subject to considerable preanalytical variables. Low levels of antithrombin, protein C and protein S occur in a variety of circumstances and test results and the clinical implications of both positive and negative results may be misinterpreted.

A full blood count and routine screening coagulation tests may be helpful. The APTT may detect some lupus anticoagulants (LAs), but only if a lupus-sensitive reagent is used. The thrombin time will detect heparin contamination and the prothrombin time (PT) is useful as an indicator of liver synthetic function for the interpretation of protein C and protein S levels. Testing is usually delayed until at least 1 month after completion of a course of anticoagulation with a vitamin K antagonist or DOAC. Testing should be avoided during intercurrent illness and use of COCPs or HRT should be noted. Sometimes testing is performed during pregnancy and so interpretation must be made with this in mind.

Functional assays should be used where the accuracy and precision are acceptable. However, no single method will detect all defects. For example, a protein C chromogenic assay will not detect a dysfunctional protein C molecule with impaired phospholipid binding due to a mutation in the Gla domain. A clot-based protein C assay would be sensitive to this defect, but imprecision of the assay would result in reduced sensitivity and specificity for other defects compared with a chromogenic assay. Using APTT-based assays will result in erroneous low protein C levels in the presence of factor V Leiden and other causes of activated protein C resistance. Similarly, the performance of antithrombin assays will be influenced by heparin concentration, the preincubation time with heparin, use of FXa or bovine or human thrombin as a substrate. For example, an assay utilising a short heparin incubation time will detect heparin binding-site defects, which may not be associated with an appreciably increased risk of venous thrombosis.

Even in families with characterised defects, a phenotypic assay may fail to accurately discriminate affected and non-affected individuals.

Preanalytical variables

Most assays are affected by clots in the sample. Antibody assays may be affected by atypical antibodies such as paraproteins, rheumatoid factor and heterophile antibodies. Chromogenic assays are subject to interference by lipaemia and haemolysis. Clot-based assays are subject to imprecision due to variation in the baseline clotting time and the levels of other coagulation or anticoagulant factors.

Low levels of antithrombin, protein C or protein S may relate to age, sex, acquired illness or drug therapy. Interpretation of results in children must be with reference to the normal range for age.

Low levels of antithrombin, protein C or protein S suspected to be the result of heritable mutations should be confirmed on one or more separate samples. Demonstrating a low level in other family members supports a diagnosis of heritable deficiency and characterisation of the genetic mutation can confirm the diagnosis and give some indication of thrombosis risk, particularly in the case of antithrombin deficiency.

Antithrombin assays

Activity assays typically use a chromogenic substrate in which a synthetic peptide is linked to *p*-nitroaniline. Cleavage of the peptide releases the *p*-nitroaniline with a resultant colour change. Patient plasma is incubated with heparin and then an excess of thrombin or FXa is added. The heparin-activated antithrombin is complexed and neutralises some of the thrombin or FXa. The remaining free protease quantified by cleavage of the appropriate chromogenic substrate is inversely proportional to the antithrombin level in patient plasma.

Interference by other proteases and inhibitors in the plasma sample is a potential problem. Interference by heparin cofactor II, another heparin-activated serpin, can be eliminated by using FXa or bovine thrombin as the target protease, as heparin cofactor II only inhibits human thrombin. The effect of other proteases in the patient plasma can be minimised by addition of a protease inhibitor such as aprotonin.

The total amount of antithrombin protein can be measured immunologically, for example by ELISA. As antithrombin antigen levels may be normal or near normal in type II deficiency, immunological assays may fail to identify patients with these variants.

Antithrombin levels are slightly reduced in infants, during pregnancy, in women taking oestrogen preparations, and in some patients during heparin therapy. Levels also decrease with age and acquired low levels occur in liver disease, DIC, nephrotic syndrome, during L-asparaginase therapy, and in protein malnutrition and cachexia.

Protein C assays

Protein C defects are divided into type 1, with a reduced amount of functionally normal protein, and type 2, where there is reduced activity due to a dysfunctional protein. In type 2 cases, there is discordance between the functional and antigenic levels. The laboratory diagnosis of protein C deficiency is based on a functional assay. Most commercially available functional assays of protein C employ the specific activator Protac, a

protein C activator derived from the venom of the southern copperhead snake (*Agkistrodon contortrix*). Chromogenic assays using a synthetic substrate for APC are simple to perform and can detect all type 1 defects and also type 2 defects affecting the catalytic site. A chromogenic assay will not detect most other type 2 defects. Clotting assays utilising the prolongation of either the PT or APTT after Protac activation have the capacity to detect other, but not all, type 2 defects, but are subject to imprecision due to variation in the baseline clotting time and the levels of other factors, for example if there is a short APTT due to a high FVIII level, or the presence of the factor V Leiden mutation which results in a falsely low protein C activity due to APC resistance, or in the presence of a LA. The diagnosis of protein C deficiency is problematic because of the wide overlap in protein C activity between heterozygous carriers and unaffected individuals. Protein C levels are not affected by pregnancy or oestrogen exposure. Acquired low levels of protein C occur during anticoagulant therapy with oral vitamin K antagonists, vitamin K deficiency, DIC and liver disease.

Protein S assays

Protein S is usually quantified immunologically rather than measured functionally. Originally, free protein S was measured in plasma supernatant after precipitation of C4b-bound protein S by polyethylene glycol (PEG). Nowadays PEG precipitation is not required as monoclonal antibodies that detect only free protein are used. Functional protein S assays are imprecise and are not used in the majority of coagulation laboratories.

Protein S levels are significantly lower in premenopausal females, so much so that different reference ranges are required for males and females and if this is not done there is a significant risk of a false-positive diagnosis of protein S deficiency in women. Protein S levels fall progressively during normal pregnancy and are reduced by oestrogens. Acquired low levels of protein S occur during anticoagulant therapy with oral vitamin K antagonists, vitamin K deficiency, DIC and liver disease.

FVR506Q and APC resistance

Nowadays, the *FV*R506Q mutation is usually detected by direct genetic analysis. APC resistance assays typically utilise the APTT. Samples are tested with and without an added fixed concentration of APC. The clotting times are expressed as a sensitivity ratio, i.e. the APTT (seconds) of patient plasma plus APC divided by the APTT (seconds) of patient plasma without added APC; the lower the ratio, the greater the APC resistance. Platelet contamination and activation must be avoided as this will lead to apparent APC resistance. The APC resistance assay is abnormal not only in the presence of *FV*R506Q, but with all other causes of heritable and acquired APC resistance and in individuals with a prolonged baseline APTT due to clotting factor deficiencies or anticoagulant therapy. It is therefore not specific for *FV*R506Q. Modification of the test by a one in four predilution of the test plasma in FV-deficient plasma increases the sensitivity and specificity of the assay for *FV*R506Q to almost 100% and this test is reliable if genetic analysis is not available.

*F2*G20210A

A laboratory clotting test with sensitivity and specificity for this mutation is not available and so detection is by direct genetic analysis.

General recommendations for laboratory tests and interpretation

- Testing at the time of acute venous thrombosis is not indicated.
- Testing is most easily interpreted when the patient does not have any circulating anticoagulant. Oral vitamin K antagonists cause a reduction in protein C and S levels. Dabigatran results in overestimation of AT, PC and PS levels and variable observations have been made around the effect of rivaroxaban, apixaban and edoxaban on different types of antithrombin, protein C and protein S assay.
- Functional assays should be used to determine antithrombin and protein C levels.
- Chromogenic assays of protein C activity are less subject to interference than clotting assays and are preferable.
- Immunoreactive assays of free protein S antigen are preferable to functional assays.
- If an APC resistance assay is performed to detect *FV*R506Q, the modified APC sensitivity test should be used. If positive, the mutation should be confirmed by a direct genetic test. An APC resistance assay is unnecessary if a direct genetic test for FV Leiden is used initially.
- Repeat testing for identification of deficiency of antithrombin, protein C and protein S is indicated and a low level should be confirmed on more than one sample. Deficiency should not be diagnosed on a single abnormal result.
- Thrombophilia testing must be performed by experienced laboratories with appropriate quality control and assurance. Results should be interpreted by an experienced clinician considering the clinical circumstances and all relevant factors that may influence individual test results.

Acquired risks for thrombosis

Pregnancy and venous thromboembolism

Around 1 in 1000 pregnancies is complicated by DVT or pulmonary embolism. Widespread use of thromboprophylaxis in at-risk pregnancies has contributed to massive pulmonary embolism no longer being the leading cause of pregnancy-related death in the UK. However, it still remains a major cause of death in the Triennial Confidential Enquiry. Although

the postpartum phase is the time of highest risk, it is shorter and overall at least as many episodes of VTE occur antepartum, including some in the first trimester. There are notable clinical features in pregnancy-related DVT: around 70% of thromboses affect the deep veins of the left lower limb and involve the iliofemoral vein from the time of presentation; thrombosis restricted to the deep veins of the calf is uncommon in pregnancy. The left-sided predominance may relate to the anatomical relationship between the iliac artery and vein on that side. Many affected women develop post-thrombotic syndrome.

The pathogenesis of pregnancy-related VTE is multifactorial. Pre-existing patient factors, such as age and obesity, along with specific pregnancy-related changes in coagulation such as suppressed systemic fibrinolytic capacity and the progressive increase in plasma concentrations of coagulation factors from early pregnancy including factor (F)VIII and fibrinogen, along with the fall in plasma concentration of the anticoagulant cofactor protein S all contribute to thrombosis risk. Complications of pregnancy such as multiple pregnancy, hyperemesis gravidarum and pre-eclampsia along with operative delivery, especially emergency caesarean section also contribute to the risk.

Diagnosis of VTE in pregnancy is dependent on imaging in all cases because the use of pre-test probability scores, such as the Wells score combined with D-dimer assays, has not been validated in pregnancy. In cases of suspected pulmonary embolism, the use of bilateral Doppler ultrasound of the legs negates the need for radiation exposure such as V/Q or CTPA scanning if it is positive. This is relevant as CT pulmonary angiography is associated with an increased risk of breast cancer in the mother of around 10%. V/Q scanning delivers a lower dose of radiation to the mother but may carry a slight increased risk of childhood cancer; in both situations, the absolute risk is very small. V/Q SPECT has a similar sensitivity and specificity to CTPA.

The treatment of choice for pregnancy-associated VTE is with low-molecular-weight heparin (LMWH). This is based on the low rate of complications and side effects and the comparative ease of administration. Dosing should be based on the woman's booking or early pregnancy weight. There is insufficient evidence to recommend whether the dose of LMWH should be given once daily or in two divided doses. Treatment with therapeutic doses of subcutaneous LMWH should be continued during the remainder of the pregnancy and for at least six weeks postnatally and until at least 3 months of treatment has been given in total. Once labour is established LMWH should be discontinued; where delivery is planned, either by elective caesarean section or induction of labour, the last therapeutic dose of LMWH should be at least 24 hours prior to planned delivery. Regional anaesthesia and analgesic methods should not be undertaken until at least 24 hours have elapsed after a therapeutic dose of LMWH. In addition, LMWH should not be recommenced for at least 4 hours after the use of spinal anaesthesia or after epidural catheter removal, and epidural cannulae should not be removed within 10–12 hours of the most recent dose of LMWH.

There is evidence of an association between heritable thrombophilia and pregnancy morbidity, including pregnancy loss, pre-eclampsia and intrauterine growth retardation. It has been shown however that antepartum prophylactic dalteparin does not reduce the occurrence of pregnancy loss or placenta-mediated pregnancy complications in pregnant women with thrombophilia at high risk of these complications and is associated with an increased risk of minor bleeding. Routine use of LMWH as VTE prophylaxis in all women with positive thrombophilia testing is of dubious benefit and should be confined to those with previous or family history of VTE.

Immobility and venous thromboembolism

It is likely that venous stasis contributes to the pathogenesis of some cases of VTE. Sitting still for 90 min reduces the flow through the popliteal vein by 50%. Immobility is probably one of many contributors to hospital-acquired VTE and is almost certainly a factor in the pathogenesis of DVT in paralysed and immobilised limbs.

There has been considerable interest in the relationship between VTE and prolonged immobilisation during travel. The term 'economy class syndrome' has been coined to encompass the occurrence of VTE during and after air travel. Epidemiological data suggest a relative risk of around twofold, which persists for 2–6 weeks after the index journey. A similar level of risk applies to other modes of powered travel, including by road. The incidence of fatal pulmonary embolism induced acutely by air travel is less than one in a million journeys. In addition, there is little evidence for any increased thrombosis risk in journeys of less than 4 hours of continuous travel. Unsurprisingly older travellers with recent surgical or medical admission and those with a previous history of VTE or other known risk factors for VTE are at highest risk. The contribution of hypobaric hypoxia to hypercoagulability is not proven and as such it appears that for travel-associated VTE immobility is the key risk factor. It is reasonable to assume that any form of long-distance powered travel carries a low, but slightly increased, risk of VTE and to recommend simple precautionary measures such as maintenance of mobility in order to maintain venous flow in the lower limbs. In patients with overt risk and in those with a previous travel-associated thrombosis pharmacological thromboprophylaxis may be considered for journeys over 4 hours.

Hospital-acquired thrombosis

VTE is common in patients in hospital and in those who have been recently hospitalised. One of the best illustrations of this comes from the Million Women's Study 2009 which demonstrated a 70-fold increased risk of subsequent VTE in women undergoing surgery and a 10-fold increased risk of thrombosis in

patients undergoing more minor day procedures compared with no procedure. Patients admitted to medical wards also have a higher thrombosis risk during and post-discharge. This has led to the widespread adoption of thromboprophylaxis in this group. This intervention has significantly reduced thrombosis occurrence. Hospital-acquired thrombosis (HAT) may occur up to 90 days after admission, with the highest incidence of DVT in the first week and highest incidence of PE in the second to third week after discharge. The pathogenesis is multifactorial, immobility, active malignancy and the acute phase response seen in patients with inflammatory disorders and sepsis all contribute. The highest incidence of VTE follows major lower limb orthopaedic surgery. In hip replacement and knee replacement surgery, subclinical DVT is frequently detectable by imaging, although there is evidence that clinically significant VTE may be becoming less common due to improvements in surgical techniques and materials, as well as wider use of thromboprophylaxis. Extended prophylaxis for up to 28–35 days has been shown to reduce VTE in those undergoing hip replacement and abdominal or pelvic surgery for cancer. Patients undergoing total knee replacement benefit from 14 days of pharmacological prophylaxis. For those undergoing hip or knee replacement, several direct oral anticoagulants (DOACs) along with unfractionated and low molecular weight heparins are licenced for thromboprophylaxis. Several studies have shown good prevention of VTE post orthopaedic surgery using an initial period of either a DOAC or LMWH followed by oral aspirin and aspirin monotherapy is advised post-total knee replacement by NICE in England.

Indwelling venous devices

The use of indwelling venous catheters is common in patients with malignancy, acute renal failure and those requiring total parenteral nutrition and critical care. Thrombosis of these lines is common with a higher incidence observed in patients with large-diameter lines and those with a tip positioned distal to the superior vena cava. A number of other factors contribute to the thrombosis risk, including infection, extrinsic compression, the use of certain types of chemotherapy and a previous history of VTE. Many studies have tried to address the role of thromboprophylaxis in prevention of catheter-associated thrombosis. Systematic reviews remain vague on the benefits of thromboprophylaxis with slight reductions in thrombotic events being off-set by bleeding. Treatments include local thrombolysis and anticoagulation with or without line removal.

Pharmaceuticals and vaccines

Combined oral contraceptive

VTE associated with use of the combined oral contraceptive (COCP) is well documented. The overall risk of VTE in a COCP user is around four to fivefold higher than in a non-pill user but this is affected by the progestogen content of the preparation. The risk is increased by a family history of thrombosis, obesity, thrombophilia and older age (see Figure 48.1). Fortunately, the absolute thrombosis risk remains acceptable due to the low background incidence of thrombosis in women of childbearing age. Combined pills induce a state of activated protein C resistance, and this appears to be more marked with the third-generation contraceptives than second-generation preparations. Most preparations in current use contain an equivalent dose of oestrogen, usually 30–35 μg of ethinylestradiol. However, the progestogen content varies. In second-generation pills, this is levonorgestrel, whereas in third-generation products it is desogestrel, gestodene or norgestimate. There is convincing epidemiological evidence that third-generation pills carry a greater risk of clinical VTE. Progestogen-only oral contraceptives appear to carry a lower risk of VTE such that their use can be considered in women in whom the COCP is contraindicated.

Hormone-replacement therapy

Oral hormone-replacement therapy (HRT) is also associated with a VTE risk. The relative thrombosis risk is equivalent to second-generation COCPs, but the absolute risk is greater due to the higher background incidence of VTE in older women. Observational data indicate a very high rate of VTE in women with a previous history of venous thrombosis who subsequently embark upon HRT. Such is the level of risk that HRT is generally contraindicated in such women. If there are overwhelming indications for HRT (disabling menopausal symptoms or gender reassignment) some clinicians consider anticoagulation alongside HRT in high-risk situations. The thrombosis risk associated with oral HRT is not apparent with transdermal preparations. It is suggested that this is due to the avoidance of the first-pass effect in the liver. Studies also suggest a higher thrombosis risk associated with the use of oral conjugated equine oestrogen compared with oestradiol.

Heparin-induced thrombocytopenia

Heparin-induced thrombocytopenia (HIT) is an important diagnosis because failure to recognise the syndrome carries a high risk of morbidity and mortality. It is due to development of antibodies directed against a complex of heparin and platelet factor 4. It is more commonly seen with unfractionated heparin (UFH) than LMWH and in surgical rather than medical or obstetric patients. Typically, the platelet count begins to fall between 5 and 10 days after first exposure to heparin. In subjects with previous recent exposure, it may occur earlier and very occasionally the thrombocytopenia develops after heparin has been discontinued. The condition may complicate heparin administered at any dose. In contrast to other drug-related immune thrombocytopenias, the degree of thrombocytopenia is rarely severe ($<20\times10^9$/L), and bleeding is not a common feature. There may be systemic symptoms, including fever and a local reaction at the heparin injection site, such as erythema or even skin necrosis after subcutaneous

administration. Because of platelet activation, there is often new thrombosis, and this may manifest before the platelet count reaches thrombocytopenic levels. When heparin has been administered to treat arterial disease the new thrombosis is most commonly arterial, and in treatment of DVT, there is usually extension of the presenting thrombus, often with pulmonary embolism. Thrombotic stroke, myocardial infarction, visceral ischaemia and DIC have all been reported.

Confirmatory laboratory tests are not entirely satisfactory. Immunoassays for heparin/platelet factor 4 are sensitive, but lack specificity because a significant proportion of subjects treated with heparin have positive antibody tests, but neither thrombocytopenia nor thrombosis. In contrast, bioassays are specific, but high sensitivity is difficult to achieve. Few centres provide a reliable bioassay for routine diagnosis.

The most practical approach to diagnosis outside the intensive care unit, where thrombocytopenia is common due to the other causes that may coexist with HIT, is the use of a pre-test probability scoring system based on the timing of onset of thrombocytopenia, presence or absence of new thrombosis, presence or absence of other potential cause of thrombocytopenia, and the degree of thrombocytopenia (the four Ts score). If the probability score is high or intermediate, heparin should be stopped and an alternative non-cross-reacting anticoagulant commenced while further investigation is carried out. An alternative anticoagulant such as argatroban, danaparoid or fondaparinux must be administered immediately as the risk of thrombosis persists for several days after heparin withdrawal. There are small case series where DOACs have been used in patients who can swallow. The outcomes as might be predicted have been satisfactory. When a high or intermediate score is combined with the results of an immunoassay including high-dose heparin neutralisation, this results in improved sensitivity and specificity for the diagnosis of HIT. The antibody may cross-react with low-molecular-weight heparins and they should be avoided. The pathogenic antibodies tend to become unmeasurable in the ensuing few months, but further exposure to heparin should be avoided in the long term. Patients with a diagnosis of HIT who present without a thrombotic event should be anticoagulated with a non-heparin anticoagulant for at least 30 days.

Vaccine induced immune thrombocytopenia and thrombosis

Vaccine induced immune thrombocytopenia and thrombosis (VITT) was first described early in 2021 when the vaccination programme for SARS-CoV2 started to take place on a large scale. The details around this condition and its management are still emerging. VITT describes an aberrant immune response to vaccines against SARS CoV2 which results in the formation of anti-PF4/vaccine antibodies. In a similar fashion to HIT, the antibodies bind to and activate platelets through their Fc receptor and this results in a catastrophic pro thrombotic illness. In distinction to HITT, these antibodies are not heparin dependent. The incidence of VITT is around 14 per million vaccine doses. The key features are thrombosis at unusual sites with associated features of a consumptive coagulopathy including thrombocytopenia, hypofibrinogenaemia and high levels of D-dimers. The most commonly affected sites are the cerebral veins but thrombosis is also observed in the splanchnic circulation as well as in heart, lungs and limbs. Thrombosis may be arterial or venous. Suspected cases are identified from their history of vaccination, with most patients presenting between 5 and 40 days after a first dose of vaccine. The complication is much more common in recipients of the two adeno-associated virus (AAV) vaccines (Astrazeneca ChAdOx1 nCoV-19 and Johnson and Johnson Ad26.COV2.S) but has been reported following receipt of non-AAV vaccines. Following clinical suspicion, based on the presentation and vaccine history it is usual for blood testing and scanning to be performed. The presence of thrombosis along with thrombocytopenia, hypofibrinogenaemia and very elevated D-dimers increase the likelihood of VITT. The majority of cases will have a positive anti-PF4 ELISA which may remain elevated for some time. It is worth noting that there is variability in the sensitivity of different anti-PF4 assays to VITT antibodies.

Treatment of VITT includes early therapy with IVIgG, high-dose steroids and anticoagulation with a non-heparin anticoagulant. Plasma exchange and rituximab have been used. The optimal duration of anticoagulation in these cases is not currently known. The mortality rate for this condition is high at around 25% with many patients succumbing very rapidly following diagnosis to a thrombotic or haemorrhagic death but may be falling with improved awareness and experience.

Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is an acquired thrombophilic condition. The essential features are arterial or venous thrombosis or recurrent pregnancy loss or placental dysfunction occurring in a subject in whom laboratory tests for antiphospholipid antibody are persistently positive. Diagnostic criteria have been established in order to standardise diagnosis for the purposes of clinical studies (Table 48.2).

DVT, pulmonary embolism and ischaemic stroke are the principal thrombotic manifestations, but any vessel may be involved, including the microvasculature. The full range of clinical features is not necessarily present. For example, women can have severe thrombotic events without pregnancy complications whilst those with pregnancy complications commonly have no other manifestation. In addition to thrombosis and pregnancy failure, additional clinical and laboratory features are variably present in APS. These include mild thrombocytopenia and livedo reticularis. Cardiac valvular abnormalities occur in up to

30% of patients but are usually subclinical. Exceptionally, the syndrome may manifest as widespread multiorgan microvascular occlusion, with multiorgan failure, so-called catastrophic APS.

In primary APS, the syndrome presents in isolation while APS may occur in association with other chronic systemic autoimmune diseases, usually systemic lupus erythematosus (SLE) when it is termed secondary APS.

Antiphospholipid antibodies

Antiphospholipid antibodies (APA) react with proteins that bind negatively charged phospholipids. These may be detected in solid-phase ELISA assays or in coagulation-based tests. The most important is β_2-glycoprotein I (β_2-GP1), as pathogenic antibodies are most frequently reactive with this protein. β_2-GPI is a member of the complement control protein family. It has five domains but antibodies reactive to a specific epitope on domain I are most pathogenic.

The LA is an *in vitro* phenomenon in which the presence of the APA slows phospholipid-dependent coagulation tests. This is probably due to impaired assembly of the components of the prothrombinase complex. LA antibodies are usually reactive to β_2-GPI/phospholipid or to prothrombin/phospholipid. LA due to prothrombin-reactive antibodies may be negative in anticardiolipin assays. Subjects with APS may have any combination of LA and anticardiolipin positivity.

Pathogenic mechanisms in APS

Important candidate mechanisms for thrombosis are antibody-induced enhanced thrombin generation, interference with the activated protein C anticoagulant pathway, and increased monocyte and endothelial tissue factor expression. Platelet activation and inhibition of fibrinolysis by antiphospholipid antibodies have also been proposed. In relation to pregnancy failure, it is noteworthy that early miscarriage before full placentation is common. This may be attributed to antiphospholipid antibodies causing inhibition of early trophoblast growth. A role for complement activation in pregnancy loss has also been proposed. Placental dysfunction is the hallmark of the second- and third-trimester complications of intrauterine fetal death, intrauterine growth restriction, pre-eclampsia and placental abruption.

Diagnosis of antiphospholipid syndrome

As indicated in Table 48.2 the diagnosis of APS is based on the presence of a qualifying event combined with the demonstration of a persistently positive APA test over a period of at least 12 weeks. Transient antibodies may occur after infection, syphilis being the prime example, and may be seen in association with several drugs particularly chlorpromazine.

Laboratories should perform both solid phase and coagulation-based assays in the assessment of suspected cases of APS. Overall, it appears that LA is associated most strongly with clinical events. In relation to solid-phase assays, high-titre antibodies and IgG antibodies associate more strongly with clinical manifestations than do IgM and lower-titre antibodies. Patients who are positive for LA, anticardiolipin and anti-β2 GP1 form a group termed triple positive APS. Emerging data indicate a more severe disorder with higher rates of thrombosis recurrence. Anticoagulation of this group is discussed below.

Table 48.2 Diagnostic criteria in APS.

*Clinical criteria**
- Thrombosis: arterial, venous or microvascular thrombosis in any tissue or organ

Pregnancy complications
- Unexplained death of morphologically normal fetus at or beyond 10 weeks of gestation
- Three or more unexplained consecutive miscarriages before 10 weeks
- One or more premature births of a morphologically normal fetus before 34 weeks of gestation due to pre-eclampsia, eclampsia or severe placental insufficiency

*Laboratory criteria**
- Antiphospholipid antibody
- IgG or IgM anticardiolipin antibodies at moderate or high concentration†; IgG or IgM anti-β_2-GPI >99th percentile *and/or*
- Lupus anticoagulant

* There must be at least one clinical and at least one laboratory criterion present. The laboratory test must be consistently positive on at least two occasions 12 weeks apart as transient antibodies may occur, for example in infection. Such antibodies are not usually associated with clinical events.

† Evidence of the definition of moderate/high concentration. In general, values of IgG anticardiolipin >40 GPLU are considered to be moderate-titre antibodies; >99th percentile has been employed also. The significance of low-titre antibodies is less clear.

Lupus anticoagulant tests

The characteristics of LA are that they produce prolongation of phospholipid-dependent tests that are not due to factor deficiency or specific factor inhibitors. These features are determined by repeating abnormal phospholipid-dependent tests in the presence of normal plasma and in the presence of excess phospholipid. Lupus inhibitors will produce prolongation of the test which is not corrected by normal plasma but is corrected by an excess of phospholipid. Other inhibitors, like the Xa inhibitors and anti-factor VIII inhibitors, will not correct with addition of plasma or phospholipid. The tests most frequently employed to detect LA are the activated partial thromboplastin time (APTT), the dilute Russell's viper venom time (DRVVT) and, less frequently the kaolin clotting time (KCT) or silica clotting time (SCT).

Because no LA test shows 100% specificity and sensitivity, more than one test system should be used for the detection of LA. The PT and thrombin time should also be performed as

they are not usually affected by the presence of LA and the results assist in the interpretation of LA tests.

APTT reagents have varied sensitivity to LA and are often labelled as LA sensitive or not by manufacturers. The specificity of the APTT for inhibitor detection is improved by inclusion of a mixing study with platelet-free normal pooled plasma but very few labs routinely do this. Inhibitors to coagulation factors, usually FVIII, also cause prolongation of the APTT which may not be corrected by addition of normal plasma. Typically, FVIII inhibitors are time and temperature-dependent, unlike LA. Diagnostic confusion may arise due to LA that prolongs the APTT, causing erroneously low results in all APTT-based coagulation factor assays.

Russell's viper venom directly activates factor X and in the presence of Factor V, prothrombin, calcium and phospholipid leads to cleavage of fibrinogen to form a fibrin clot. As such, inhibition of the phospholipid, along with various factor deficiencies and the presence of acquired and pharmacological inhibitors of the pathway will produce prolongation of the coagulation time. The process of repeating the tests in the presence of normal plasma and in excess of phospholipid allows interpretation of the assay. As with all coagulation tests, because of variations in reagents and techniques, it is essential that laboratories derive local normal ranges and participate in external quality assessment.

The SCT is used in some centres as a screening test for LA. Assays with low levels of phospholipid appear to be the most sensitive and positive results can be confirmed by using an excess of phospholipid to demonstrate phospholipid dependency of the test. The KCT is like an APTT with no additional phospholipid however there is no phospholipid correction test. The test is affected by clotting factor deficiencies and anticoagulants but is rarely used partly due to problems with automation of the assay.

Alternative tests for LA may be employed. They include the tissue thromboplastin inhibition test and clotting tests that use venoms other than Russell's viper venom. An example is the Taipan venom: this test has been shown to have a strong positive predictive value in patients receiving warfarin.

Solid-phase assays (for anticardiolipin and anti-β_2-GP1 antibodies)

The other methods for detecting antiphospholipid antibodies involve solid phase assays such as anticardiolipin and more specific anti-β_2-GP1 assays. The introduction of international standards has allowed the calculation of anticardiolipin results in IgG or IgM antiphospholipid units (GPLU and MPLU, respectively) related to a given concentration of affinity-purified anticardiolipin immunoglobulin. Despite this, issues remain around the precision, and accuracy of these assays. Interpretation of the anticardiolipin assay is not straightforward and in patients positive for LA and anticardiolipin assays the tests may not be identifying the same antibodies. The clinical significance of low-titre anticardiolipin is doubtful but may be difficult to interpret in patients who have a qualifying clinical event for APS.

Specific assays for anti-β_2-GPI antibodies have been developed, and several commercial kits are available. Anti-β_2-GPI antibody assays may show higher precision and better correlation with the thromboembolic complications in APS and SLE than assays for anticardiolipin and are less likely to give transient positive results in association with infection.

Management of APS

Thrombosis

Data from observational studies suggest that the recurrence rate, following a first episode of otherwise unprovoked VTE, in an individual who is diagnosed with APS is high enough to warrant long-term anticoagulation. In patients at equipoise post-VTE, investigation into APS is probably the single most relevant 'thrombophilia test'. Previously, higher INR targets had been set for these patients but it has been shown that an INR target of 2.5 is suitable for most APS patients. The anticoagulant of choice in APS-related thrombosis has become much debated following changes to the SPC of the DOACs in relation to their use in patients with APS. Expert groups continue to debate the interpretation of the statement in SPCs that 'DOACs are not recommended for patients with a history of thrombosis who have a diagnosis of APS' which evolved following a 2019 study, TRAPS, that showed an excess of thrombotic events in patients with APS randomised to rivaroxaban as opposed to warfarin with an INR target of 2.5. Whether the results of this study, where most of the thrombosis was arterial, commute to patients with venous thrombosis is the issue. Clinical trials in patients with arterial thrombosis and APS have been smaller and less convincing as a result.

Immunosuppressive therapy is not indicated in primary APS. An exception may be catastrophic APS when combination treatment with antithrombotics, corticosteroids and other immunomodulatory therapies such as immunoglobulin and plasma exchange is administered as a potentially life-saving emergency measure.

Pregnancy

It is important that the criteria in Table 48.2 are adhered to when dealing with obstetric APS. Miscarriage is a common occurrence and up to 3% of women of childbearing age will have antiphospholipid antibodies which, in most are of no known relevance. In women with recurrent first-trimester miscarriage as defined, around 15% will have APA although the pathogenicity of these is not wholly clear. A small proportion of women with antiphospholipid antibodies also have anti-Ro antibodies. Their detection is important as anti-Ro is associated with a 2% risk of complete heart block in the fetus and a 10% chance of neonatal lupus.

The management of a pregnancy in a mother with obstetric APS includes enhanced ante-natal care with increased surveillance

by ultrasound scanning and in most cases the use of the antithrombotics LMWH and aspirin. The use of aspirin and LMWH has become usual practice in many centres and countries despite the lack of convincing evidence for its benefit. Many women who present to obstetric clinics have a pre-formed opinion on what their management should be based on their own initial research and many feel that the addition of antithrombotic therapy may improve their chances of a live birth.

Thromboprophylaxis for those with APS and a previous history of thrombosis is based on the use of LMWH, although there is no international consensus on dosing.

Venous thromboembolism and cancer

The association between cancer and venous thromboembolism is well established. Thrombotic events are common and rank second only to the direct effects of the cancer as a cause of death in cancer patients. In addition to intrinsic biological features of the primary disease, there are other factors, including surgery, chemotherapy, radiotherapy, hormonal therapies and the use of indwelling central lines that increase thrombosis risk in this group of patients. The development of venous thromboembolism in the context of malignancy is generally a poor prognostic feature often being indicative of advanced and aggressive disease. Several mechanisms may contribute to the propensity to develop venous thromboembolism in the context of active cancer and some of these may also arise as consequences of the use of some forms of chemotherapy. Proposed mechanisms include activation or disruption of endothelial function, activation of coagulation and platelets and inhibition of natural anticoagulants. Amongst the best-documented mechanisms are the expression of tissue factor and cancer procoagulant on tumour cells and the prothrombotic properties of mucins secreted by adenocarcinomas. There is evidence that tissue factor expression, which may result from proto-oncogene expression and tumour-suppressor gene inhibition, confers a proangiogenic state, which may enhance the aggressiveness and invasiveness of cancers. Several tumour types including lung, brain, pancreas, stomach, ovary, renal and lymphoma have the strongest reported association with thrombosis development. Whether this is related to the direct effects of thrombosis or reflects inherent tumour aggressiveness is not clear. Cancer chemotherapy may result in an excess of both venous and arterial thrombosis. An excess of VTE is seen with the use of platinum-based drugs cis- and carboplatin, with the iMIDs, thalidomide, lenalidomide and pomalidomide, with anti EGFR antibodies cetuximab, panitumumab and necitumumab and with the CDK inhibitors palbociclib, abemaciclib and ribociclib. Use of bevacizumab and the VEGFR TKIs sunitinib, sorafenib and axitinib is associated with an excess of arterial thrombosis, while the *BCR::ABL* TKIs dasatinib, nilotinib and ponatinib are associated with an excess of both arterial and venous thrombosis.

Cancer patients with VTE have poorer outcomes in terms of the need for hospital admission and higher rates of major bleeding and thrombosis recurrence on anticoagulant therapy, compared with patients who do not have cancer. It is now usual practice for the majority of patients with active cancer who are admitted to hospital in the UK to receive thromboprophylaxis during their stay. The role of routine thromboprophylaxis in ambulatory cancer patients receiving chemotherapy is less clear. A Cochrane review of nine RCTs involving 3538 patients, which compared patients receiving thromboprophylaxis (eight LMWH and one warfarin) with control groups found a reduction in VTE risk without a significant increased risk of bleeding. However, the number needed to treat to prevent one episode of thrombosis was 60, suggesting that further stratification to identify those individuals at the highest risk was needed. In a more recent Cochrane review in 2021 of 10 RCT, 6 on the use of VKA and 4 on the use of DOACs there was no reduction in mortality in the treated groups. Risk assessment scores such as that developed by Khorana, which identified patients with stomach, pancreas, lung, lymphoma, gynaecologic, bladder and testicular cancer combined with a platelet count $>350 \times 10^9$/L, haemoglobin <100 g/L, leucocyte count $>11 \times 10^9$/L and a body mass index of >35 kg/m^2 may or may not be helpful.

The initial management of established venous thromboembolism in patients with active cancer differs from usual therapy. Several studies have shown that continuous treatment with LMWH at therapeutic doses compares favourably with the use of coumarins with reduced thrombosis recurrence without any increase in major bleeding. The reasons for this difference in efficacy of LMWH are not clear, but it has been suggested that better compliance with therapy, lower rates of drug interactions and less interference around times of poor or limited oral intake (when vitamin K intake is variable) may contribute. The management of patients with cancer with an ongoing indication for anticoagulation after 6 months is less clear and a variety of options, including LMWH, warfarin and DOACs are used. Patients, however, find ongoing compliance with parenteral therapy difficult and uncomfortable. Two studies have investigated the option of replacing LMWH with a DOAC after an initial period of treatment. SELECT D compared rivaroxaban with LMWH showing a reduced rate of recurrent VTE but a slight excess of bleeding while ADAM VTE compared LMWH with apixaban showing a significant reduction in VTE recurrence with no excess of bleeding in favour of apixaban. Although it has been suggested that heparins improve life expectancy through an anti-cancer effect, recent data do not support this suggestion.

Because of the association between cancer and thrombosis various studies have investigated the potential benefit of extensive cancer screening in patients with, what appear to be, unprovoked events. This has included the use of tumour biomarkers

and extensive CT scanning. Most studies show that while extensive investigation may lead to slightly earlier cancer diagnosis in some patients, it does not affect outcomes of the cancer. This is reflected in many subsequent guidelines.

Thrombotic risk in myeloproliferative disease (polycythaemia rubra vera and essential thrombocythaemia) (see also Chapter 35)

Untreated, polycythaemia vera (PV) affects life expectancy, with the majority of patients dying of vascular occlusion, which may involve large vessels or the microvasculature. There is clearly significant overlap between PV and essential thrombocythaemia (ET), and thrombosis may involve both arterial and venous circulation. There does appear to be a predisposition towards thrombosis in the cerebral circulation, but events occur elsewhere and notably involve the splanchnic vessels. A well-described entity is the development of unprovoked intra-abdominal venous thrombosis in individuals who have the *JAK-2* V617F mutation without the typical peripheral blood phenotype of MPD.

In PV there is good evidence that the incidence of vascular occlusion is positively related to the packed cell volume, with the lowest incidence in patients with good control of haematocrit. The role of thrombocytosis in risk of thrombosis in PV is controversial. The risk of thrombosis in PV increases with advancing age, a past history of thrombosis, and presence of other risk factors for thrombosis. Thrombosis risk is reduced by control of haematocrit and antiplatelet therapy.

The risk of thrombosis in ET is related to patient age and the presence of other risk factors for thrombosis. Recent data indicate an increased thrombosis risk in patients with ET who are *JAK-2* V617F positive and in those with elevated white cell counts. Platelet count appears more closely related to bleeding than thrombosis.

The microvascular events and vasomotor manifestations of both PV and ET may relate to quantitative and qualitative changes in red cells and platelets, as they are not seen in other forms of polycythaemia or in reactive thrombocytosis. The role of white cells and endothelial cells that form part of the clone is not clear. It is possible that similar small-vessel occlusive and vasomotor changes seen in the feet and hands may also occur in other parts of the body. Erythromelalgia, a syndrome consisting of painful burning red extremities with normal peripheral pulses, is the characteristic vasomotor disturbance. Livedo reticularis is occasionally seen. In the digital vessels, the development of thrombosis can lead to ischaemia and gangrene. In relation to the cerebral circulation, a range of symptoms and signs, including transient cerebral ischaemia, transient monocular blindness, migraine, headaches and seizures are seen. Both the cerebrovascular complications and erythromelalgia of myeloproliferative disease may respond promptly to low-dose aspirin and/or cytoreduction.

Inflammation and thrombosis

The activation of the coagulation system is appropriate as a defence mechanism in the context of bacterial and viral infection where clot formation can limit the dissemination of pathogens via the bloodstream. In terms of evolution, it is therefore not surprising that there are numerous interactions and 'cross talk' between infection, inflammation and thrombus formation pathways. However, the aberrant or excessive activation of so-called thromboinflammation resulting in immunothrombosis may result in pathological clot formation in both the venous and arterial systems. Evidence for this association is supported by the increased number of cardiovascular events seen in patients with inflammatory disease and following both serious and apparently more minor infection. It is quite likely that this activation of inflammatory pathways (Table 48.3) underlies the observation of many hospital-acquired thromboses, discussed earlier. The main cellular drivers of immunothrombosis are innate immune cells, especially monocytes and neutrophils. These drive a process of cell activation that results in activation of inflammatory cytokines (interleukin-6 and 1β), the coagulation cascade and complement. Inflammation upregulates procoagulant factors, downregulates physiological anticoagulants and inhibits fibrinolytic activity and ultimately when defence systems such as DNase 1 and activated protein C are swamped, thrombosis results. It is likely that there are differences between the process of thrombosis formation as a result of thromboinflammation in venous and arterial vessels. However, it does seem that the activation of both platelets and neutrophils and their subsequent interaction is required for both. Nuclear extracellular traps (NETs) are found in both arterial and venous thrombi. They are formed in response to microbial

Table 48.3 Effects of inflammation on haemostasis.

Increased
- Tissue factor expression
- Neutrophil and monocyte activation
- NETs formation
- Surface procoagulant activity, negatively charged phospholipid
- Neutrophil/Platelet interactions
- Platelet activation
- P-selectin expression
- Levels of fibrinogen and other coagulation proteins
- PAI-1

Decreased
- Thrombomodulin expression
- Endothelial cell protein C receptor
- Half-life of activated protein C
- Fibrinolytic activity
- Endothelial glycosaminoglycans

infection and to other endogenous danger signals such as those resulting from inflammation. They have a significant role in clearing large pathogens but may also result in the formation of thrombi in the vascular bed. NETs formation, NETosis, has many triggers but the binding of P-selectin to P-selectin glycoprotein ligand-1 (PSGL1) on neutrophils is one of the best recognised and may represent a potential therapeutic target. NETs can activate the coagulation cascade by providing a negatively charged surface to allow contact activation and by inhibition of the natural anticoagulant TFPI.

The protein C anticoagulant pathway appears to be highly influenced by inflammation. Thrombomodulin and endothelial cell protein C receptor are both downregulated by inflammatory cytokines and the overcoming of the protective effect of the aPC pathway may result in thrombosis formation.

Endothelial cell activation causes multiple prothrombotic changes, including downregulation of fibrinolysis by increased production of plasminogen activator inhibitor (PAI-1) which may be one of the key factors in the prothrombotic state associated with COVID-19.

An excess of venous thrombosis is seen in patients with chronic inflammatory conditions including inflammatory bowel disease, Behçet disease, rheumatoid, SLE and diabetes. The exact pathogenesis of VTE is not clear in these conditions but almost certainly involves some of the mechanisms discussed above. In patients with SLE, NETs degradation is impaired resulting in persistent activation of complement.

Atherosclerosis can be considered an inflammatory state and the recent description of atherosclerotic plaque formation associated with clonal haematopoiesis of indeterminant potential (CHIP), allows an explanation of the possible mechanisms involved. The acquired myeloid gene mutations, *DNMT3A*, *TET2*, *ASXL1* and *JAK2* are all associated with an increased rate of coronary artery disease. It is likely that the expression of these pro-inflammatory mutations in monocytes results in the formation of active plaque.

The recognition of the associations between inflammation and thrombosis has triggered an appropriate interest in exploring the potential for immunosuppression and anti-inflammatory drugs as an intervention to prevent thrombosis. Several studies involving canakinumab, methotrexate and colchicine have reported with the key issue being the balance between a reduced rate of thrombosis and increased rates of infection associated with the intervention.

Haematological prothrombotic states due to non-malignant diseases of the blood and bone marrow

Paroxysmal nocturnal haemoglobinuria (see Chapter 31)

Paroxysmal nocturnal haemoglobinuria is a rare acquired disorder that results from mutations in the phosphatidylinositol glycan class A (*PIG-A*) gene. This results in the production of blood cells that lack the normal mechanisms to regulate the effects of complement. As many as 40% of patients with an eventual diagnosis of PNH will have suffered a venous thrombosis prior to diagnosis and 30–45% will suffer from an event in the course of their disease. Thrombosis is the commonest cause of death, accounting for 40–65% of PNH deaths. Thrombosis occurs at many sites, but there is an excess of events in the splanchnic circulation and in the cerebral veins. Occasionally, painful discoloured skin lesions occur when superficial dermal veins are affected. These lesions rarely ulcerate. Occasionally, skin lesions can resemble purpura fulminans; these can affect large areas of skin with necrosis and demarcation. In pregnancy, PNH is associated with an increased risk of fetal loss (40%) as a result of thrombosis and haemorrhage. The prothrombotic state in PNH is complex and multifactorial. This includes platelet activation, the effects of intravascular red cell haemolysis and neutrophil and monocyte activation. Platelet and endothelial microparticles from activated cells along with oxidative stress may also be relevant. Impaired fibrinolysis has also been observed and may be caused by interactions between complement activation, coagulation and fibrinolysis. Anticoagulation of patients with PNH has typically been with a VKA but there is no obvious reason why DOACs could not be used in this regard. There is ongoing discussion around the need for anticoagulation when the PNH clone size has been minimised, which can occur in patients treated with eculizumab or ravulizumab.

Thrombotic thrombocytopenic purpura

See Chapter 46.

Sickle cell disease (see Chapter 15)

Sickle cell disease is complicated by microvascular and macrovascular occlusion with resultant tissue ischaemia distal to the sickling. Venous thromboembolism is a problem in patients with sickling conditions. Several studies have shown a cumulative incidence of VTE between 11 and 17% by the age of 40 years. Recurrence rates are also high at around 35% at 5 years. The rate of VTE in patients with sickle cell disease is higher than that seen in other frequently hospitalised patients with other chronic disorders. The exact mechanisms that result in an excess of VTE in patients with sickle cell disease are not clear and are likely multiple. There is evidence of a prothrombotic state shown by increased levels of prothrombin fragment 1 + 2 and thrombin–antithrombin complexes. Sickled erythrocytes adhere more readily to vascular endothelium and strongly accelerate coagulation due to abnormal exteriorisation of procoagulant anionic membrane phospholipids. Recently the P-selectin inhibitor Crizanlizumab has been shown to reduce vaso-occlusive crisis in patients with sickle cell and it will be interesting to see whether this will translate into a reduced incidence of VTE. Other interventions between P-selectin and P-selectin glycoprotein ligand are current causes of interest.

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CHAPTER 49

Prevention and management of venous thromboembolism

49

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Introduction

Venous thromboembolism (VTE), which comprises deep vein thrombosis (DVT) and pulmonary embolism (PE), has an incidence of about 1 per 1000 per annum, with two-thirds presenting as DVT and one-third as PE. Approximately half of VTEs are associated with hospital admissions, and hospital VTE prevention programmes are important to help reduce this risk. The case fatality of VTE is approximately 5%, but there is also major morbidity caused by post-thrombotic syndrome (PTS) of the leg and chronic thromboembolic pulmonary hypertension (CTEPH). Anticoagulants are highly effective in secondary prevention, but leave the patient at risk of bleeding. The risk of recurrence is considerable and patients need to be assessed to identify those who require long-term anticoagulation as secondary prevention. This chapter covers primary VTE prevention (risk assessment and thromboprophylaxis), VTE diagnosis and acute treatment, and secondary VTE prevention.

VTE prevention

Approximately half of VTEs are associated with hospital admissions. Thromboprophylaxis has been shown in clinical trials to reduce the risk of VTE by approximately 50% for medical inpatients and up to 70–80% following orthopaedic surgery.

A national VTE prevention programme was launched in England in 2010 to reduce this hospital associated morbidity and mortality. The foundation of the programme was national guidance and standards for VTE prevention (National Institute of Clinical Excellence, NICE, guidance). This guidance requires all adults (over the age of 16 years) to be VTE risk assessed on admission to hospital using a standardised risk assessment tool. If a patient is assessed to be at increased risk of VTE without significant bleeding risks, the patient is advised to have pharmacological thromboprophylaxis daily whilst an inpatient. Surgical patients have historically been additionally considered for mechanical thromboprophylaxis; however, recent clinical trial evidence from the GAPS trial challenges this concept as it demonstrated that anti-embolism stockings did not provide additional reduction in VTE compared to low molecular weight heparin alone in elective surgical patients. A few select groups of patients are also considered for extended thromboprophylaxis on discharge (total hip and knee replacement, major abdominal cancer surgery).

England's VTE prevention programme requires hospitals to record the number of patients who have a VTE risk assessment on admission to hospital (target greater than 95%); and to review all cases of hospital-associated-thrombosis (defined as VTE occurring within 90 days of hospitalisation) to check whether or not the patient received appropriate thromboprophylaxis daily. If on review it is considered that the patient did not receive appropriate thromboprophylaxis each day, then the hospital-associated-thrombosis is reported as 'potentially preventable'. A 'potentially preventable' hospital-associated-thrombosis reflects that an error occurred. Formal root cause analysis of 'potentially preventable' thromboses is recommended in order to identify the causes of the error and

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
© 2025 John Wiley & Sons Ltd. Published 2025 by John Wiley & Sons Ltd.
Companion website: www.wiley.com/go/mead/HPH8e

then action changes to reduce the risk of such an error occurring again. Evaluating the impact of this national VTE prevention programme is challenging but there is evidence of a reduction in 'potentially preventable' hospital-associated-thromboses and mortality associated with VTE since it was introduced.

Choice of VTE risk assessment

All VTE risk assessments are based on both VTE risk factors and bleeding risk factors. If a person is identified as being at risk of VTE without bleeding risk factors, then pharmacological thromboprophylaxis is prescribed. If a significant bleeding risk factor is identified then pharmacological thromboprophylaxis is not offered, and mechanical thromboprophylaxis alone may be considered.

The majority of hospitals in the UK currently use the National VTE risk assessment tool (Department of Health) 2010 although this tool has not been validated in randomised clinical trials. In this tool, the presence of a single additional VTE risk factor in a surgical patient or in a medical patient with reduced mobility is considered to indicate a high VTE risk, with the presence of any bleeding risk factor indicating a high bleeding risk (see Table 49.1). Pharmacological thromboprophylaxis is recommended for those with a high VTE risk and low bleeding risk, with consideration of mechanical thromboprophylaxis alone in those with both high VTE risk and high bleeding risk.

Various VTE risk assessment tools have been reported in the literature including the Padua Prediction score, the predictive (four factor) IMPROVE score, the Geneva risk score, the Khorana score and the Caprini risk assessment model. The Padua Prediction Score is currently the best-validated score for inpatients. It gives a weighted score and patients who score 4 or more are considered at high risk of VTE and considered for pharmacological thromboprophylaxis (see Table 49.2)

Choice of anticoagulant and dose

The initial landmark trials of thromboprophylaxis in medical inpatients (MEDENOX, PREVENT) used a prophylactic dose of low molecular weight heparin (LMWH). This remains the recommended pharmacological thromboprophylaxis for medical inpatients. For people with a contraindication to heparin such as a history of heparin-induced thrombocytopenia, allergy or those who refuse medications of porcine origin, alternatives such as fondaparinox can be used. There is evidence to suggest that the dose of LMWH should be increased in obesity, and so many hospitals advise weight-based prophylactic LMWH regimens.

Prophylactic doses of the Direct Oral Anticoagulants (DOACS) Apixaban and Rivaroxaban were shown to be safe and effective alternatives to LMWH following total hip and knee replacement and are licenced as an alternative pharmacological prophylaxis agent following these operations.

Duration and extended thromboprophylaxis

The majority of guidelines currently advise to consider pharmacological thromboprophylaxis only as an inpatient except after certain high VTE risk operations: 10–14 days post total knee

Table 49.1 Table of additional VTE risk factors and bleeding risk factors, based on the National VTE risk assessment (Department of Health).

Additional VTE risk factors	Bleeding risk factors
• Age over 60 years, • Active cancer or cancer treatment • Critical care admission • Dehydration • Known thrombophilia • Obesity (BMI over 30 kg/m^2) • One or more significant medical comorbidities (for example): heart disease, metabolic endocrine or respiratory pathologies, acute infectious diseases and inflammatory conditions • Personal history of VTE or first- degree relative with a history of VTE • Use of hormone replacement therapy • Use of oestrogen-containing contraceptive therapy • Varicose veins with phlebitis • Pregnancy or less than 6 weeks postpartum	• Active bleeding • Acquired bleeding disorder (such as acute liver failure) • Concurrent use of anticoagulants known to increase the risk of bleeding (such as warfarin with an INR 2 or more; direct/novel oral anticoagulants such as apixaban, rivaroxaban, edoxaban, dabigatran; or fondaparinux) • Acute stroke • Thrombocytopenia (platelets less than 75×10^9/L) • Uncontrolled systolic hypertension (230/120 mmHg or higher) • Untreated inherited bleeding disorder (such as haemophilia and von Willebrands disease) • Lumbar puncture/epidural/spinal anaesthesia within the next 12 hours • Lumbar puncture/epidural/spinal anaesthesia within the previous 4 hours • Other high-risk bleeding procedure such as neurosurgery, spinal surgery or eye surgery

Table 49.2 Padua VTE risk assessment model (high risk of VTE: ≥4).

Score	Baseline characteristic
3	Active cancer*
3	Previous VTE (with the exclusion of superficial vein
3	thrombosis)
3	Reduced mobility**
2	Already known thrombophilic condition***
1	Recent (<1 month) trauma and/or surgery
1	Elderly age (≥70 years)
1	Heart and/or respiratory failure
1	Acute myocardial infarction or ischaemic stroke
1	Acute infection and/or rheumatologic disorder
1	Obesity (BMI ≥30)
	Ongoing hormonal treatment

* Patients with local or distant metastases and/or in whom chemotherapy or radiotherapy had been performed in the previous 6 months.
** Bedrest with bathroom privileges (either due to patients' limitations or on physicians' order) for at least 3 days.
*** Carriage of defects of antithrombin, protein C or S, factor V Leiden, G20210A prothrombin mutation, antiphospholipid syndrome.

replacement; 28–35 days post total hip replacement, and 28 days following major abdominal cancer surgery.

In order to reduce the burden of VTE associated with hospitalisation, large randomised controlled trials have looked at prophylactic LMWH or low-dose DOAC as extended thromboprophylaxis following medical hospitalisation (for 28–35 days post-discharge). Meta-analysis of these trials showed a reduction in symptomatic VTE or VTE death (0.8 versus 1.2%, $p = 0.002$) but with increased major or fatal bleeding (0.6 versus 0.3%, $p < 0.001$). Given this balance of risk and benefit, the American College of Chest Physician Guidelines do not currently recommend extended thromboprophylaxis for medical patients.

Ambulatory thromboprophylaxis

Patients with certain cancers, particularly pancreatic cancer, have a greatly increased risk of thrombosis. All patients should be advised of the signs and symptoms of VTE and to seek medical attention early if they experience them. Risk stratification tools have been designed with the aim of risk stratifiying ambulatory patients who are at very high risk of VTE, and offering thromboprophylaxis. However primary thromboprophylaxis in this setting is generally not currently recommended.

Compared to when the original landmark trials of medical and surgical thromboprohylaxis were performed, the average duration of medical and surgical hospitalisations have become shorter. This combined with advances in surgical techniques and support of early mobilisation post-operatively may have reduced the baseline risk of thrombosis, changed the balance of risk/benefit, and is an area for further research. Furthermore, medicine in the UK is increasingly moving towards ambulatory, same-day emergency care with supported early discharge; research is required to examine the VTE risk in this relatively new and expanding medical cohort in order to ascertain whether or not (a subgroup of) these patients may benefit from thromboprophylaxis.

Diagnosis of VTE

Diagnosis of deep vein thrombosis

It is difficult to diagnose a deep vein thrombosis (DVT) by clinical means alone. A patient can present in a 'classical' way – with a unilateral swollen, hot and painful calf. However, symptoms and signs are highly variable and the contemporary diagnostic pathway for a patient who presents with symptoms suggestive of a deep vein thrombosis (DVT) is to follow a diagnostic algorithm (see Figure 49.1), in recognition of this diagnostic uncertainty, which combines clinical, laboratory and radiological tools.

Algorithms for the diagnosis of a deep vein thrombosis

The objective diagnosis of DVT depends on diagnostic imaging, using either a three-point compression or a duplex ultrasound scan (USS). These methods are reported to have a 97–98% sensitivity. However, because of the increasing number of negative tests, strategies have been developed that can exclude the diagnosis in some patients without the need for imaging. These strategies rely on the use of information from the clinical history and examination to estimate a pretest probability (PTP) and assays to detect D-dimers. The commonest PTP probability assessment tool is the Wells score (see Table 49.3). An 'unlikely' PTP score is not on its own enough to rule out a DVT, but an unlikely score can be used to rule out the diagnosis if the D-dimer test is also negative (Figure 49.1).

Thrombus formation is followed by a fibrinolytic response, which releases fibrin degradation products (containing the D-dimer motif) into the circulation. It follows that absence of a rise in D-dimers implies that thrombosis is not occurring. The strategy for using D-dimer assays in the diagnosis of DVT is to employ a sensitive test with a high negative predictive value. Fully quantitative, laboratory D-dimer tests are recommended above point-of-care D-dimer tests in the UK, but point-of-care tests can be used where laboratory testing is not available, to speed up diagnosis. The specificity of the assays for DVT is poor and false positive results are common.

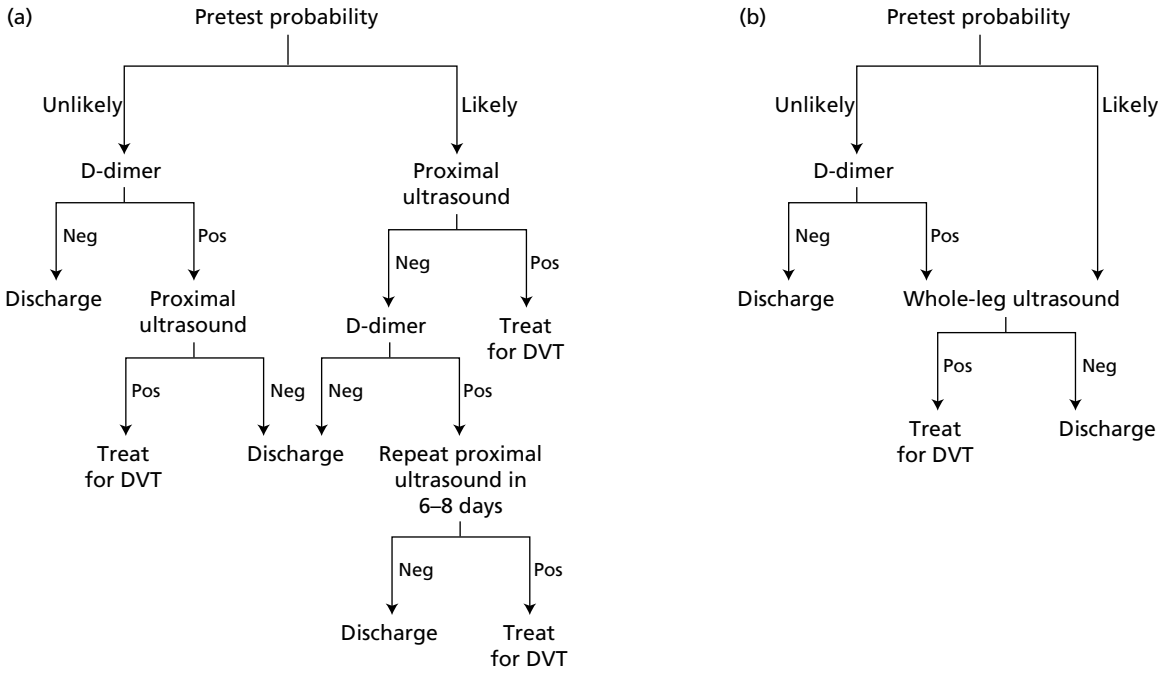

Figure 49.1 (a) Diagnostic algorithm for DVT with scanning of proximal veins only. (b) Diagnostic algorithm for DVT with scanning of whole leg.

Table 49.3 Wells clinical score used for predicting the probability of DVT prior to further testing.

Risk factors, symptoms or signs	Points
Active cancer (patient receiving treatment for cancer within the previous 6 months or currently receiving palliative treatment)	1
Paralysis, paresis or recent plaster immobilisation of the lower extremities	1
Recently bedridden for 3 days or more, or major surgery within previous 12 weeks	1
Localised tenderness along the distribution of the deep venous system	1
Entire leg swollen	1
Calf swelling at least 3 cm larger than the asymptomatic leg (measured 10 cm below tibial tuberosity)	1
Pitting oedema confined to the symptomatic leg	1
Collateral superficial veins (non-varicose)	1
Previously documented deep vein thrombosis*	1
Alternative diagnosis at least as likely as deep vein thrombosis	−2

Score ≥2 points, likely pretest high probability; score ≤1-point, unlikely pretest probability.
* The authors give a point for previous DVT or PE.

All patients who have a 'likely' PTP or who have a raised D-dimer need to proceed to diagnostic imaging. This is illustrated in Figure 49.1a, b.

There are two accepted algorithms where a patient who requires an USS either undergoes a whole leg scan or has a proximal scan only – which looks only at the venous system as far as the popliteal veins. A whole leg scan needs to be performed only once, whereas if a patient has a first proximal scan that is negative, but has a raised D-dimer, they will need a repeat scan a week later, principally to exclude proximal extension of a distal DVT. If only the proximal veins are scanned, we know that patients with an 'unlikely' PTP and those with a normal D-dimer do not need a second scan. Scanning the calf veins (e.g. in a whole leg scan) increases the complexity of the scan and the duration of the procedure. The evidence on whether, or not, to treat distal DVTs is uncertain (see below).

There is some debate about which cut-off to use for the D-dimer value within the DVT diagnostic algorithm. Traditionally, the manufacturer's cut-off has been used. However, it is well known that D-dimer levels rise with age and there are prospective observational data for DVT, and retrospective data for PE, that support the use of age-adjusted D-dimer cut-offs, without reducing sensitivity of the diagnostic algorithms. In the UK, the National Institute for Clinical Excellence (NICE) provided a recommendation in 2020

suggesting that age adjustments in D-dimer test thresholds could be used for people aged over 50. (An age-adjusted D-dimer is calculated as follows: Age (years) × 10 for patients >50 years. For example, for a patient aged, 76 – a threshold of 760 ng/mL FEU would be applied).

Isolated calf DVT

DVT usually starts in the calf, but by the time symptoms develop most patients have thrombus in the proximal veins. If isolated calf DVT is left untreated, extension into the proximal veins has been reported in 3–18%, usually within one week. Isolated calf DVTs that do not extend rarely lead to clinically significant emboli and it is safe to withhold anticoagulant treatment from patients with clinically suspected DVT who have normal compression ultrasonography of the proximal veins at the time of presentation and one week later. This led to the two different ultrasound strategies for DVT diagnosis set out above (Figure 49.1). If the whole leg is scanned and an isolated calf DVT found, it is oftentreated, though, in the absence of severe symptoms or risk factors for extension, the American College of Chest Physicians suggests serial imaging instead of initial anticoagulation – which in practical terms involves an USS weekly for a further two weeks after diagnosis, and if no proximal extension is seen, then anticoagulation is not required. However, these recommendations do have caveats and if a patient has any of the following higher risks, standard anticoagulation is recommended by the American College of Chest Physicians: elevated D-dimer, severe pain, known history of cancer or previous VTE, the patient is hospitalised, or the DVT is more than 5 cm long or is unprovoked.

High-quality evidence to support treatment for distal DVT is weak. The only RCT looking at distal DVT evaluated 259 patients and was terminated early due to slow recruitment with only 50% of the required sample size. Patients were randomised to 6 weeks of treatment dose nadroparin or placebo. There was no difference (3% nadroparin versus 5% placebo) in the composite endpoint of extension to proximal DVT, presence of contralateral DVT or symptomatic PE within 42 days of study start. There was in addition a greater risk of bleeding (4 versus 0% patients) with treatment.

It must be remembered that the diagnostic algorithm is heavily based on the results of a D-dimer test. Therefore, the algorithm is useful only in the out-patient setting, since D-dimers will be elevated in most in-patients. The limitations of USS must also be recognised. USS are excellent tools for diagnosing DVT in the leg but are more limited for DVT that are more proximal: e.g. the iliac veins and indeed the inferior vena cava (IVC). If a patient presents with a significantly swollen leg, up to the groin and the USS is negative – a CT venogram should be considered to rule out a more proximal clot. In a similar way, if a patient complains of bilateral swollen legs, a CT venogram can diagnose an IVC clot.

Diagnosis of pulmonary embolism

Clinical presentation of PE depends on the size, location and number of emboli, and the patient's underlying cardiorespiratory reserve. The classic triad of chest pain, haemoptysis and dyspnoea is present in less than 20% of patients, but nearly all will have at least one of pleuritic chest pain, dyspnoea or tachypnoea.

Diagnostic imaging is necessary to make the diagnosis and CT pulmonary angiography (CTPA) and ventilation–perfusion (V/Q) lung scanning are both used. Perfusion scanning with technetium-99-labelled albumin macroaggregates is a sensitive, but not very specific, method. The specificity is improved by adding ventilation scanning in which a gaseous radionuclide such as xenon-133 or technetium-99 diethylenetriaminepentaacetic acid (DTPA) is inhaled in an aerosol form, looking for a V/Q mismatch (no perfusion in ventilated lung). However, V/Q scanning is often non-diagnostic, resulting in a non-informative 'intermediate probability' scan report. CTPA, which may also identify alternative diagnoses, is most commonly used.

In order to reduce the need for diagnostic imaging a similar strategy to that used for DVT is applied. The initial step for patients presenting with a possible PE is also to assess them for their pretest probability using a scoring system such as the Wells PE score (Table 49.4). Patients scoring as 'unlikely' can have the diagnosis excluded without the need for diagnostic imaging if they also have a negative D-dimer. If, however, the D-dimer is positive, the patient should be sent for diagnostic imaging, as should all patients who have a 'likely' PTP (Figure 49.2). Similarly, to DVT, an age-adjusted D-dimer may be used for patients over the age of 50 years.

Table 49.4 Wells clinical score used for predicting the probability of PE prior to further testing.

Risk factors, symptoms or signs	Points
Signs and symptoms of DVT	3
Alternative diagnoses less likely than PE	3
Heart rate >100	1.5
Immobility or surgery within 4 weeks	1.5
Previous DVT/PE	1.5
Haemoptysis	1
Active cancer	1

Score >4 points, likely pretest probability; score ≤4 points, unlikely pretest probability.

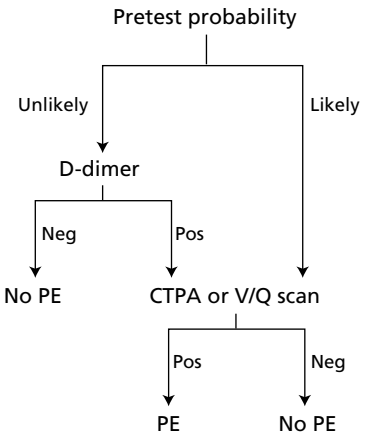

Figure 49.2 Diagnostic algorithm for PE.

Treatment of VTE

The majority of deaths (>90%) due to PE occur in untreated patients in whom the diagnosis is not made in life. Treatment with therapeutic dose anticoagulation reduces the mortality to approximately 2%, with half of deaths directly attributable to PE rather than comorbidity, such as cancer. Patients with symptomatic PE are 5 times more likely to die of fatal PE than patients with symptomatic DVT and patients with massive PE with systemic hypotension (high-risk PE) are 10–20 times more likely to die. Rapid commencement of anticoagulation, and thrombolysis in patients with high-risk PE, is therefore required to prevent death. In patients presenting with DVT without symptomatic PE, death is unusual once treatment with anticoagulation has started.

Blood samples for haemoglobin, platelet count, PT and APTT, serum creatinine and liver function should be taken. A group and save should also be ordered for patients who will be treated with thrombolysis. Thrombophilia testing is of no utility at this stage, as the results do not affect immediate patient management with any drug. Only those patients who offer a history, or have clinical signs, suggestive of an underlying malignancy should be referred for further appropriate investigation.

Initial anticoagulant therapy

Once a diagnosis of DVT or PE is made, or strongly suspected, a rapidly acting anticoagulant should be started immediately. The most commonly used anticoagulants are the direct oral anticoagulants (DOACs) which include the anti-Xa inhibitors (e.g. apixaban and rivaroxaban) and the direct thrombin inhibitor, dabigatran. The advantage of two of the anti-Xa inhibitors, namely apixaban and rivaroxaban, is that they can be given without any concomitant low-molecular-weight heparin (LMWH) in the first days after diagnosis. All other DOACs require a cross-over treatment period of at least 5 days with LMWH followed by continuation of the DOAC alone.

Not all patients with a diagnosis of VTE can be treated with a DOAC. Care should be taken using DOACs in patients with renal impairment, and depending on the specific drug, a DOAC should not be used when the creatinine clearance falls below 15–30 mLs/minute. A patient's weight should be considered – the clinical trial data for DOACs did not include a large number of overweight patients (weight greater than 120 kg, or BMI greater than 40 kg/m^2). However, recent guidance in 2023 from the ISTH supports the use of DOACs in patients weighing more that 120 kg due accumulating real-world data supporting their clinical effectiveness in this group. Patients with triple positive antiphospholipid syndrome should be offered warfarin as their first-line treatment option.

Another area of some uncertainty is the use of DOACs for unusual site VTE. The clinical trial data mostly relates to the efficacy of DOACs for DVT and PE treatment and therefore some clinicians are more hesitant using DOACs for other indications. As is often the case, not only are real-world data emerging but also, as confidence grows in the use of DOACs, many clinicians do opt for oral medications.

For those patients who are unable to have a DOAC, a vitamin K antagonist (VKA) such as warfarin or treatment dose LMWH alone can be used. The LMWH dose is based on weight and the total daily dose is given as a single or two divided subcutaneous injections without the need for monitoring or dose adjustment. LMWH should continue for at least 5 days and until the International Normalised Ratio (INR) has been greater than 2.0 for two consecutive days when it is used in combination with warfarin.

When initiating rapid loading with warfarin, a loading nomogram should be used, as individual warfarin dose requirements vary considerably. The advantages of using a dosing nomogram are:

- A therapeutic intensity of anticoagulation is reached in the shortest possible time without an excessive risk of over-anticoagulation.
- The daily maintenance warfarin dose, which will be required after the initial loading, is predictable. This reduces the risk of over-anticoagulation and hence bleeding following initial loading, and increases the likelihood of maintaining a therapeutic level of anticoagulation.

In patients with VTE who stop anticoagulant therapy after 3–6 months, the incidence of subsequent fatal PE is approximately 0.25 per 100 patient years.

Thrombolytic therapy

Streptokinase and recombinant tissue plasminogen activator (rtPA) are widely available fibrinolytic agents. Streptokinase, a glycoprotein purified from the supernatant of β-haemolytic streptococci, in complex with plasminogen, cleaves other

Table 49.5 Stratifying pulmonary embolism by risk of death.

Haemodynamically unstable PE = high risk
Systolic blood pressure <90 mmHg or a pressure drop of ≥40 mmHg for >15 min if not caused by an arrhythmia, hypovolaemia or sepsis
About 5–10% of patients are in this high-risk group and have a risk of early death of >15%
Haemodynamically stable PE
Intermediate risk
These patients are haemodynamically stable but with evidence of right heart strain or myocardial injury. Early mortality is 3–15%
Low risk
Haemodynamically stable without evidence of right heart strain or myocardial injury. Early mortality is <1%

plasminogen molecules to plasmin. This indirect action results in complex pharmacokinetic characteristics. Furthermore, streptokinase is immunogenic, which may cause allergic reactions, as well as a reduced or absent response on repeated use or after a recent infection with streptococci. tPA has a direct enzymatic effect on plasminogen that is strongly enhanced by the presence of fibrin. Guidelines on the management of acute PE now recommend stratifying patients into high, intermediate and low risk (Table 49.5). Low-risk patients can be considered for home treatment. Patients with intermediate- and high-risk PE require hospitalisation and patients with high-risk PE should be considered for immediate thrombolytic therapy as this may reduce mortality. Whilst life-saving in the acute phase, the haemodynamic benefit of thrombolysis compared to heparin is lost after the first few days. At the present time, patients with intermediate-risk are not given thrombolytic therapy, but they should be monitored, as 25% of patients deteriorate and may require urgent escalation to thrombolytic therapy. Escalation of patients to thrombolysis in clinical trials probably explains why there has been no difference in mortality in randomised trials of thrombolysis in intermediate-risk patients. In routine clinical practice, the opportunity for escalation is lost if patients are not monitored, resulting in avoidable fatal PE.

Up to 5% of patients have massive PE with hypotension at presentation. Fatal PE occurs in >15% of patients presenting with high-risk (massive) PE, 3–15% of patients with intermediate-risk PE, and in less than 1% of patients with low-risk PE or DVT alone. Right ventricular dysfunction (RVD) is present in up to 40% of patients with acute PE and normal blood pressure, and fatal PE is twice as likely in those with RVD. In patients with RVD, biomarkers such as troponin may identify those patients at greatest risk, but the clinical utility of these measurements is not yet known. Death due to acute PE results from an acute fall in cardiac output. In patients without pre-existing heart or lung disease, the haemodynamic disturbance correlates with the extent of obstruction of the pulmonary circulation.

Contraindications to thrombolysis include active internal bleeding, a stroke within 2 months, and an intracranial process such as neoplasm or abscess. Relative contraindications include surgery within 10 days, uncontrolled hypertension and pregnancy. Haemorrhagic complications are higher in patients with a recent invasive procedure such as pulmonary angiogram or placement of an inferior vena cava (IVC) filter. There is a reported incidence of intracranial haemorrhage of approximately 2%, with higher rates in the elderly and those with poorly controlled hypertension. Up to 20% of patients suffer major bleeding. If heparin is being given, it should be stopped. Heparin therapy is started 2 hours after administration of thrombolysis.

Thrombolytic therapy after DVT increases the rate of early recanalisation but is associated with a higher risk of bleeding than with anticoagulant therapy. In clinical studies, venographic obstruction and PTS were less frequent in patients treated with streptokinase compared with anticoagulation alone, but PTS assessments in these studies were not performed using any validated instrument, and bleeding complications were much more frequent in patients treated with streptokinase. Consequently, systemic streptokinase infusions are no longer used for DVT treatment. Systemic infusion of recombinant tissue plasminogen activator (rt-PA) has limited efficacy. Clinical studies suggest that the systemic administration route may not reliably achieve a therapeutic rt-PA concentration at its target sites within the thrombus. However, major bleeding is more frequent than with anticoagulant therapy. Catheter-directed intrathrombus thrombolysis (CDT) refers to the infusion of a fibrinolytic drug directly into the venous thrombus via a multisidehole catheter, which is embedded in the thrombus using imaging guidance. This is the preferred treatment currently if thrombolytic treatment is given. However, even with low-dose rt-PA (0.5–1.0 mg/h) major bleeding occurs in about 4% of patients. The long-term benefits remain unclear and so thrombolytic therapy is not recommended routinely in patients with DVT. However, it may be considered in individual patients with symptomatic iliofemoral DVT who have all of the following:

- symptoms of less than 14 days' duration;
- good functional status;
- a life expectancy of 1 year or more;
- a low risk of bleeding.

Long-term complications of VTE

Post-thrombotic syndrome (PTS)

Following acute DVT, recanalisation occurs slowly. Residual vein occlusion is present in 50% of patients at 6 months and remains present in 25% after 3 years. Clot resolution is identical in patients with and without thrombophilia. Because of frequent incomplete clot resolution, the diagnosis of recurrent ipsilateral DVT is problematic. Of particular, clinical value in this regard is

the demonstration that in patients with previous DVT, a negative high-sensitivity D-dimer assay result safely excludes recurrence. Post-thrombotic syndrome (PTS) is chronic pain and swelling, which occurs in up to one-half of patients who have a DVT; it is typically worse at the end of the day or with prolonged sitting or standing, and better after a night's rest and leg elevation. Patients may describe heaviness in the leg, cramps, pruritis and paraesthesia. There may be oedema, induration of the skin, reddish-brown hyperpigmentation and venous ectasia. Severity of PTS can be quantified using the Villalta score. PTS can occur early or, more unusually, have a latency of up to 10 years; the cumulative frequency has been estimated as 23% at 2 years and 28% at 5 years; in 5–10%, PTS is classed as severe (this includes all those with ulcers).

A major risk factor for PTS is recurrent ipsilateral DVT. During anticoagulant treatment after DVT patients with prolonged periods of subtherapeutic anticoagulation are more likely to develop PTS. There is no evidence that prolonged anticoagulant therapy reduces the risk of PTS. This indicates that early suboptimal anticoagulation rather than duration of anticoagulation influences the development of PTS and that thrombin generation may contribute to ongoing valvular damage immediately after DVT.

Early walking after acute DVT and continued high levels of physical activity appear to reduce the likelihood of clot progression and reduce the risk of PTS. Initial randomised trials demonstrated a 50% reduction in PTS with compression stockings used for up to 2 years. However, the large SOX trial failed to show a benefit when compression stockings were used for 50% of the time compared to 'sham' stockings. Consequently, any benefit of stockings in reducing the incidence and severity of PTS after DVT is now contentious, although many patients use them for symptomatic relief early after acute DVT.

Chronic thromboembolic pulmonary hypertension (CTEPH)

Following treatment of acute symptomatic PE with either thrombolysis or anticoagulation, 85% of patients still have partial occlusion of the pulmonary circulation after 1 week. At 6 months more than 50% of patients have persistent follow-up scan defects and complete resolution does not occur in the majority of patients by 1 year. Consequently, there is the difficulty of confirming or excluding recurrent acute PE in patients who do not have a follow-up scan at completion of initial treatment. Less than 5% of patients with persistent occlusion of the pulmonary circulation after 6 months will develop CTEPH. Recurrent and previous multiple PE are significant risk factors for CTEPH, but heritable thrombophilia defects are not associated with an increased risk.

Common symptoms of CTEPH include progressive shortness of breath on exertion and marked fatigue. It is important to ask patients at their 3-month anticoagulation review whether they have persistent or worsening dyspnoea and consideration should be made for further investigation if CTEPH is suspected. Diagnosis of CTEPH can be made using a combination of assessments. A V/Q scan is the preferred screening test, where a diagnosis of CTEPH can be supported by the presence of a mismatched wedge-shaped perfusion defect. An echocardiogram can also be used as part of the screening process – looking for evidence of right heart strain. Right heart catheterisation is also used to confirm the presence of pulmonary hypertension. Patients with confirmed CTEPH should be cared for by pulmonary hypertension specialists and anticoagulation continued.

Risk of recurrence and duration of anticoagulant therapy

It is important to identify an underlying clinical condition that might have caused VTE, such as cancer, by careful history, examination and appropriate investigations. Overweight patients should be advised to lose weight and smokers advised to stop. The preferred anticoagulant treatment options for patients with active malignancy and those receiving treatment for cancer are either continued LMWH or more recent data now support the use of anti-Xa inhibitors such as apixaban or edoxaban. These medications have been shown in RCTs to be slightly better than LMWH in treating, and preventing, VTE but at the cost of conferring a higher risk of bleeding for patients. Care should be taken when considering using a DOAC for gastrointestinal or urothelial tract cancers, as these patients are at greater risk of bleeding with DOAC when compared to LMWH.

If isolated calf DVT is diagnosed it is usually treated for 3 months but 6 weeks may be sufficient for isolated calf DVT following surgery. Patients with proximal DVT or PE should be treated for at least 3 months. The decision as to whether to continue anticoagulant treatment indefinitely after a first proximal DVT or PE is dependent on the risk of recurrence without continued treatment and the perceived risk of bleeding on anticoagulation. Long-term anticoagulation is usually given after recurrent unprovoked VTE. For patients who receive long-term anticoagulation, the risk-benefit ratio of continued treatment should be reassessed at regular intervals, e.g. annually or following an episode of severe over-anticoagulation (INR >8) or bleeding.

Recurrent DVT or PE is associated with a risk of death and a greatly increased likelihood of PTS and CTEPH, respectively. Secondary prevention of VTE significantly reduces the burden of disease, but recurrent VTE is only prevented for as long as anticoagulation is continued. Therefore, there is a need to improve risk stratification for both recurrent VTE and anticoagulant-related bleeding. Following an episode of venous thrombosis, patients are 40 times more likely to suffer an event in the future than patients who have not previously suffered from venous thrombosis. However, risk varies between patient groups. Patients with VTE provoked by surgery have an

annualised recurrence rate of <1%. Patients with VTE provoked by a non-surgical factor have an annualised risk of 4% and patients with unprovoked VTE have an annualised event rate of 7%. Many experts consider an annual rate higher than 5% justification for long-term anticoagulant therapy, as the risk of future fatal PE is then likely greater than the risk of fatal bleeding on long-term anticoagulation.

Decisions regarding duration of anticoagulation should be made with reference to whether or not a first episode of VTE was provoked or unprovoked (Table 49.6). Current guidelines recommend that patients with provoked VTE receive anticoagulant therapy for 3 months. For patients with unprovoked VTE, a decision should be made after 3 months as to whether the patient should continue on anticoagulation or stop. In patients with an unprovoked first event, male sex, a high D-dimer on completion of initial anticoagulant therapy and possibly younger age predict higher rate of recurrence. Decision rules for stopping anticoagulation, which include testing for D-dimer, have been evaluated. Increased D-dimer levels following completion of anticoagulant therapy after DVT are associated with residual vein obstruction (RVO) and likelihood of recurrence is not determined by RVO when adjusted for D-dimer level. Testing for heritable thrombophilia has little, if any, clinical utility in deciding whether a patient should remain on anticoagulant therapy or not, and routine testing in order to determine the duration of anticoagulant therapy is not recommended. If it is planned to stop anticoagulation treatment in a patient who has had unprovoked proximal DVT or PE, then testing for antiphospholipid antibodies should be offered, as a positive result would argue for reconsidering long-term treatment.

A further consideration when deciding whether a patient should continue anticoagulation or not after an initial 3 months of treatment is whether the first presentation was with symptomatic PE or DVT without symptoms of PE. Whilst the risk of recurrence is the same, patients who present with an initial PE are 3 times as likely to suffer from PE if they suffer a recurrence, as compared to those whose first event is a DVT.

Table 49.6 Suggested duration of anticoagulation.

3 months	3 months then consider for long-term	Usually long-term
• First proximal DVT or PE associated with a transient risk factor* • First unprovoked isolated calf DVT (consider 6 weeks if post-surgery)	• First unprovoked proximal DVT or PE**	• Recurrent unprovoked DVT or PE • Proximal DVT or PE with a significant continuing risk factor (e.g. active cancer, antiphospholipid antibodies) and no other cause

* Transient risk factors: surgery, significant trauma e.g. fracture, plaster cast; combined pill/hormone replacement therapy; pregnancy/puerperium.
** A stronger case for continuing in: males; those with raised D-dimers after completing anticoagulation; those with PE.

Other treatments

Vena caval filters

Vena caval filters are prosthetic devices that are deployed to prevent PE in patients at high risk, but in whom standard anticoagulant therapy is contraindicated, or in selected patients with PE despite therapeutic anticoagulation. Contraindications to anticoagulation insertion might be:

- central nervous system haemorrhage;
- overt gastrointestinal bleeding;
- retroperitoneal haemorrhage;
- massive haemoptysis;
- stroke in the last 14 days;
- central nervous system trauma;
- severe thrombocytopenia.

Filter placement is associated with potential complications. Because anticoagulation is usually contraindicated, at the time of filter insertion, there is a risk of thrombosis at the vascular access site. However, most of these are asymptomatic. There are no data on the long-term consequences of this complication. Mechanical problems such as caval penetration, filter migration, tilting and filter fracture are infrequent asymptomatic events with symptomatic events occurring in about 1% of patients.

With or without subsequent anticoagulation, there is a significant incidence of subsequent thrombosis in the vena cava or leg veins resulting in PTS in approximately 20%. This is not significantly different from the incidence of PTS following DVT treated with anticoagulant therapy, but due to incomplete follow-up of IVC filter patients this figure may be an underestimate. Filters are associated with a lower incidence of early PE, but there is a higher incidence of recurrent DVT and the thrombus frequently involves the inferior vena cava. Case series have not demonstrated a benefit from introducing anticoagulation for the sole purpose of preventing filter-related thrombotic events and anticoagulant therapy for patients with IVC filters should be guided by an assessment of the patient's risk of recurrent VTE and major bleeding, and not the presence of the filter alone. All efforts should be made to remove an IVC filter once a patient is able to receive anticoagulation, to minimise long-term side effects.

Thrombectomy

Thrombectomy is rarely performed in patients with massive PE but is an option in cardiothoracic centres in patients with life-threatening PE. Thrombectomy is not recommended in patients with DVT. There are insufficient data to evaluate the long-term benefit of surgical removal of the thrombus in patients.

Venous thrombosis in unusual sites

Cerebral vein thrombosis

Cerebral venous sinus thrombosis (CVST) most often affects young adults, with approximately three-quarters of patients being female. Obstruction of cerebral veins causes cerebral oedema and venous infarction, while occlusion of venous sinuses cause intracranial hypertension. It can present with recent unusual headache or stroke-like symptoms. The most sensitive diagnostic test is magnetic resonance venography. If MRI is not available, then high-resolution CT venography is useful but can be normal initially. Recognised underlying causes include infection, myeloproliferative disorders and leukaemia (especially with asparaginase treatment) and use of oestrogen-containing oral contraceptives. Mortality in the first month is approximately 5%, but 80% of surviving patients recover completely or have only a mild functional or cognitive deficit. Anticoagulant therapy was associated with a pooled relative risk of death of 0.33 (0.08–1.21) and of death or dependency of 0.46 (0.16–1.31), based on two small studies and it is recommended that patients with CVST without contraindications to anticoagulant therapy should be treated with therapeutic-dose heparin. The optimal duration of anticoagulation is unknown, but it is suggested that a minimum of 3-months treatment is given. Long-term anticoagulation has been suggested for patients with 'severe' thrombophilia and in patients with unprovoked CVST with incomplete recanalisation, but with no evidence.

Retinal vein thrombosis

Retinal vein occlusion (RVO) is predominantly a disorder of the elderly. The prognosis depends on the development of macular oedema and neovascularisation and standard therapy has been laser photocoagulation. The role of anticoagulant therapy is unclear. For branch RVO, antiplatelet or anticoagulation therapy is not recommended. Patients with acute central RVO are considered for a short course of anticoagulation with LMWH or with low-dose tPA, but routine therapy with warfarin, DOAC or antiplatelet agents is not recommended.

Upper limb DVT

Upper extremity DVT may involve the axillary, subclavian and brachial veins and may be associated with thoracic outlet syndrome, or with strenuous effort, or with placement of a central venous catheter. Compression ultrasound is an acceptable alternative to venography in the investigation of suspected upper limb DVT. PE occurs in up to 30% of cases and PTS of the arm is a risk. Recurrence is less common than in lower limb DVT. The majority of patients are managed with anticoagulation for 3 months. Those patients with a high clot burden, with a DVT involving the subclavian and axillary veins, may be considered for catheter-directed thrombolysis.

Intra-abdominal vein thrombosis

Portal vein thrombosis

The portal vein is formed from the superior mesenteric and splenic veins. The most common underlying aetiology of portal vein thrombosis is cirrhosis; other causes are intra-abdominal infection, inflammation or malignancy and blunt trauma or surgery. Myeloproliferative disorders account for up to a quarter of cases and all patients should be assessed for the *JAK2* V617F mutation. Paroxysmal nocturnal haemoglobinuria is another important cause of intra-abdominal thrombosis. Presentation can be acute, with abdominal pain, fever and nausea, or chronic, with symptoms of portal hypertension. The diagnosis can be made by CT, MRI or Doppler ultrasound. In patients with cirrhosis, the risk of anticoagulation-related bleeding is high, and anticoagulation may be regarded as too high risk. In acute portal vein thrombosis without cirrhosis, anticoagulation is usually given. There is no evidence to guide duration of anticoagulation. Although it has been suggested that anticoagulation should be given long-term in the presence of heritable thrombophilia, or if there is a failure of recanalisation, this is not based on any evidence.

Hepatic vein thrombosis

The usual presentation is with abdominal pain, ascites and hepatomegaly (Budd–Chiari syndrome). Many patients have a myeloproliferative disorder and a large proportion of patients are JAK2 positive. The diagnosis can be made with Doppler ultrasound, CT or MRI. Most patients are anticoagulated and in one-third, this is followed by transjugular intrahepatic portal-systemic shunting (TIPSS).

Mesenteric vein thrombosis

The most common causes are cancer, intra-abdominal infection, cirrhosis and surgery. Presentation is with abdominal pain and diarrhoea or peritonitis. Diagnosis is with CT or MRI. Surgery is required in patients with peritonitis, but otherwise anticoagulation is the treatment of choice.

Splenic vein thrombosis

Isolated splenic vein thrombosis is rare, and pancreatic disease is the most common aetiology. It can present with variceal bleeding and splenomegaly.

Superficial thrombophlebitis

Superficial venous thrombosis (SVT) of the lower limb, involving long or short saphenous veins or their branches, has the same risk factors as DVT. SVT was considered to be a benign and self-limiting condition; however, it is now appreciated that a significant proportion of those presenting with SVT will have concomitant DVT or PE, or are at significant risk of developing DVT or PE. SVT within 3 cm of the sapheno-femoral junction has such a high risk of progression to DVT that such patients are given therapeutic anticoagulation as for DVT. In other cases, non-steroidal anti-inflammatory drugs (NSAIDs) and heparin have been used as treatment. A Cochrane review concluded an intermediate dose of LMWH (125 units/kg) might be appropriate and for SVT ≥5 cm in length this or a prophylactic dose of fondaparinux (2.5 mg daily) for 45 days should be used, the latter reducing symptomatic DVT or PE by 82%. Rivaroxaban 10 mg daily is an alternative treatment option.

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CHAPTER 50

Pathogenesis and management of arterial thrombosis

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List of Abbreviations

ACS	acute coronary syndrome
ADP	adenosine diphosphate
AF	atrial fibrillation
aPC	activated protein C
APS	antiphospholipid syndrome
APT	antiplatelet therapy
ATP	adenosine triphosphate
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CYP	cytochrome
DAPT	dual antiplatelet therapy
DES	drug eluting stents
DIC	disseminated intravascular coagulation
DOAC	direct oral anticoagulant (also known as NOAC; non-vitamin K dependent oral anticoagulant)
EPCR	endothelial protein C cell receptor
ESC	European Society of Cardiology
GP	glycoprotein
HITT	heparin induced thrombocytopenia and thrombosis
HTPR	high on-treatment platelet reactivity
INR	international normalised ratio
LEAD	lower extremity artery disease
LMWH	low molecular weight heparin
MACE	major adverse cardiovascular events
MI	myocardial infarction
NO	nitrous oxide
NSAIDs	non steroid anti-inflammatory drugs
NSTEMI	Non ST-segment elevation myocardial infarction
OAC	oral anticoagulant
PAD	peripheral artery disease
PAI-1	plasminogen activator inhibitor 1
PAR	protease activated receptor
PCI	percutaneous coronary intervention
PCORI	Patient-Centred Outcomes Research Institute
PFO	patent foramen ovale
PPI	proton pump inhibitors
ST	stent thrombosis
STEMI	ST-segment elevation myocardial infarction
TAFIa	thrombin-activatable fibrinolysis inhibitor
TIA	transient ischaemic attack
TM	thrombomodulin
tPA	tissue plasminogen activator
TXA2	thromboxane
UFH	Unfractionated heparin

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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VITT vaccine associated immune thrombosis and thrombocytopenia
VKA vitamin K antagonist
vWF von Willebrand Factor

The impact of arterial thrombosis on cardiovascular disease

The majority of acute arterial cardiovascular diseases, like myocardial infarction and ischaemic stroke, are caused by thrombosis or thromboembolism of one or more arterial branches that provide the organ with oxygen and nutrients. Arterial thrombosis typically starts at the surface of a damaged atherosclerotic plaque and may inflict more distal obstruction through dislodgement or embolisation of thrombus material. Another distinct type of arterial thrombosis originates in association with atrial fibrillation, where thrombi form in the left atrial appendix and embolise to the brain (embolic stroke). Atherothrombotic and embolic stroke from the heart are distinctly different pathologies requiring different management strategies, discussed in the next sections.

In addition, arterial thrombosis may occur in the context of systemic hypercoagulable diseases. These include coagulopathies related to antiphospholipid syndrome (APS), malignancy and other less frequent disorders including thrombotic thrombocytopenic purpura, heparin-induced thrombocytopenia and thrombosis (HITT) or in the course of disseminated intravascular coagulation (DIC), related to systemic thrombo-inflammatory disorders, including infections like Covid-19. Finally, arterial thromboembolisation may also be a consequence of paradoxical embolisation from the venous circulation, through a patent foramen (PFO) in the heart (with a recent study suggesting such thrombi may also form within the PFO); discussion of pathogenesis and management of these less frequent conditions is beyond the scope of this chapter.

Globally, myocardial infarction and ischaemic stroke, of either origin, are the most frequent causes of cardiovascular morbidity and mortality, in either sex and in all ethnicities. Importantly, not just the primary ischaemic event, but also the consequences such as heart failure, contribute to the worldwide large-scale morbidity, characteristic for many ischaemic cardiovascular diseases.

In this chapter, we will focus on the major classes of arterial thrombotic disease, the origin of thromboembolism and their antithrombotic management.

The physiological defence against atherosclerosis

Physiologically, the vascular endothelium has important protective anticoagulant and anti-inflammatory properties. Platelets – anucleate, discoid cells produced by megakaryocytes – circulate in a non-activated state, not engaging in platelet-platelet contact. To prevent unwanted and uncontrolled platelet activation and subsequent thrombus formation, the endothelial lining of the blood vessels actively produces and secretes platelet inhibitors. Endothelial cells exposed to shear stress secrete nitric oxide (NO) continuously. Also, they produce prostaglandin I_2, a product from arachidonic acid metabolism. Both compounds provide a strong platelet inhibitory influence by elevating intracellular levels of cGMP (NO) and cAMP (PGI_2). Additionally, endothelial cells express the ectonuclease CD39, which degrades the platelet agonists ATP and ADP present in the plasma to AMP. CD73, also associated with the vasculature, subsequently converts AMP to adenosine, another platelet inhibitor that results in elevated intracellular cAMP levels. Besides the production and secretion of platelet inhibitors, the glycocalyx of the endothelium, consisting of proteoglycans and glycoproteins, provides an additional means to prevent platelet adhesion as it covers prothrombotic proteins and its negative charge repels cells.

In addition, coagulation proteases like factor (F) Xa and thrombin interact with cellular receptors. These receptors include thrombomodulin (TM) and endothelial protein C cell receptor (EPCR). Binding of thrombin to TM generates a complex that converts protein C to activated protein C (aPC), which slows down coagulation by degradation of FVa and FVIIIa and also displays anti-inflammatory and cell protective properties. The thrombin-TM complex also converts plasma pro-carboxypeptidase B2 (proCPB2 or thrombin-activatable fibrinolysis inhibitor, TAFI) to activated TAFI (TAFIa), which inhibits plasmin formation. Plasmin will normally limit fibrin clot formation and prevent thrombosis, thus TAFI formation also requires regulation to limit fibrin deposition. At the same time, TAFIa has several anti-inflammatory properties including the inactivation of pro-inflammatory mediators like bradykinin, anaphylatoxins C3a and C5a and thrombin-cleaved osteopontin (OPN). This way, physiologically, low levels of thrombin support endothelial cell integrity through endothelial cell-mediated APC-protease activated receptor (PAR) signalling and TAFIa generation.

Platelets and the pathophysiology of atherosclerosis and atherothrombosis

Endothelial cell dysfunction refers to functional endothelial impairment in response to triggers of vasodilation, but also refers to diminished anti-inflammatory and anticoagulant properties. Upon inflammatory challenge of the endothelium, PARs that physiologically help to translate the cell protective effects of low amounts of FXa and thrombin, can divert their cell signalling functions towards damaging actions, depending on cell receptor density and configuration, the concentration of coagulation factors, as well as the endothelial configuration of PARs and their cofactors. Inflammation can also result in (local) loss of TM and EPCR due to proteolysis or membrane shedding, which may (locally) diminish cell protective properties and allow endothelial cell perturbation to occur (Figure 50.1a, b).

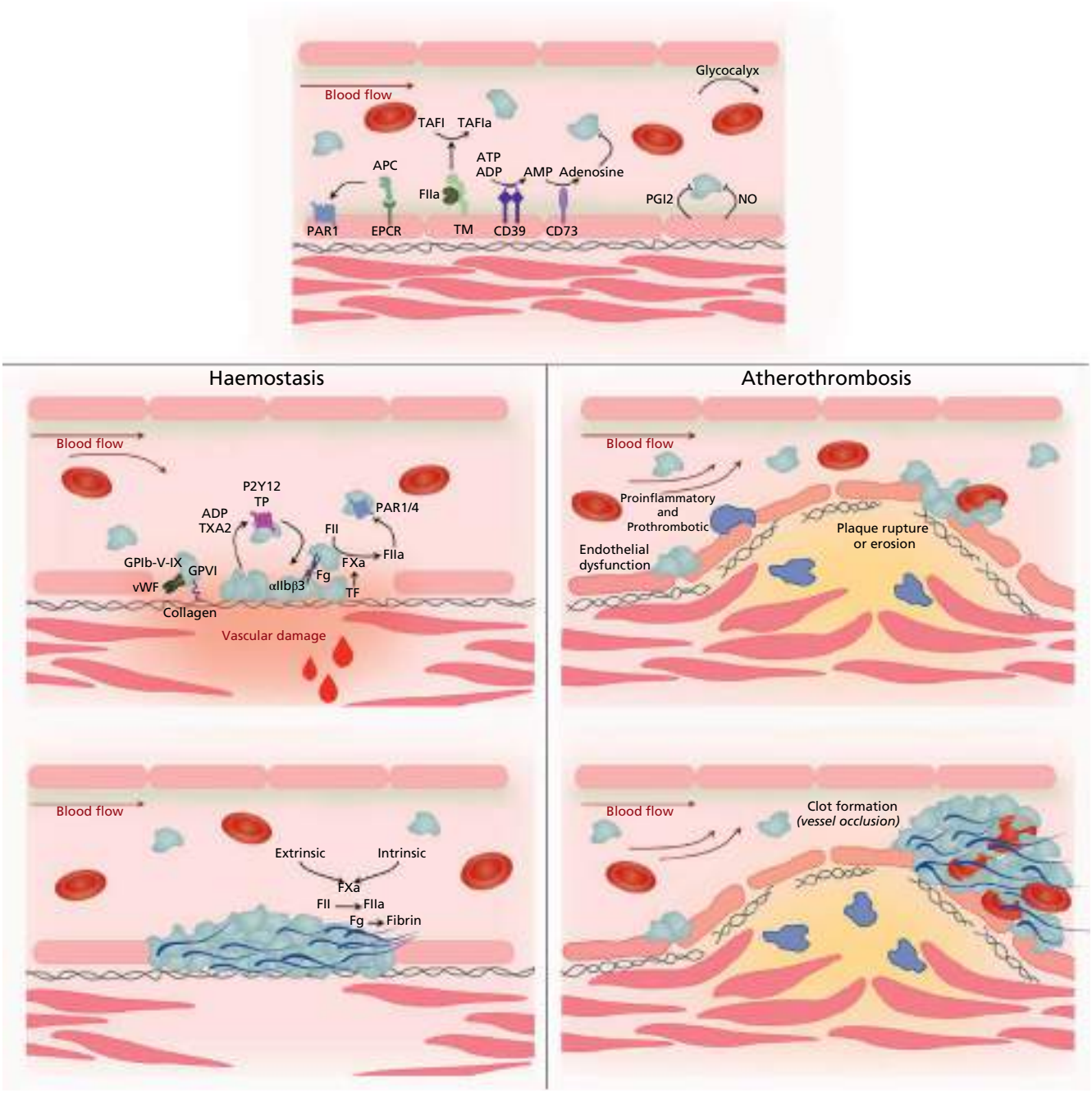

Figure 50.1 upper panel: physiological defence mechanisms of the vascular endothelium against thrombosis, showing protease-activated receptor 1 (PAR-1) and endothelial protein C receptor (EPCR) as receptors for thrombin and activated protein C, directing anticoagulant and anti-inflammatory activities. Thrombomodulin (TM) links thrombin to protein C activation as well as thrombin activatable fibrinolysis inhibitor (TAFI) activation, balancing coagulation and fibrinolysis. CD39 and CD73 direct ADP, AMP and adenosine mediated platelet reactions, while endothelial prostacyclin (PGI2) and nitrous oxide production further prevent platelet activation and act anti-inflammatory. Left middle and lower panel: the process of haemostasis occurring upon damage to the vessel wall; collagen and other platelet adhering and activating proteins allow platelet adhesion and concurrent thrombin generation further amplifies platelet activation and aggregation and thrombus formation to heal the wound bed. Right upper and lower panel: illustrate atherothrombosis occurring at the surface of a damaged atherosclerotic plaque with endothelial activation and dysfunction as early-stage events, triggering inflammation and eventually thrombosis.

When endothelial cells are activated or damaged, von Willebrand Factor (vWF) stored within the Weibel Palade bodies is released. This triggers rolling of platelets onto the damaged vascular site mediated through shear-dependent vWF – glycoprotein (GP) Iba interactions, providing platelets with the opportunity to stably adhere to other matrix components within the injured vessel or atherosclerotic plaque. Stable platelet adhesion is brought about by binding of the integrin $\alpha_2\beta_1$ and GPVI receptors to the collagen present within extracellular matrix. While platelet adhesion to collagen is widely studied, other components of the extracellular matrix, like laminin, fibronectin, vitronectin and thrombospondin, are also capable of supporting platelet adhesion and contributing to thrombus formation. Upon platelet rolling and subsequent stable adhesion to the damaged vessel wall, intracellular signalling pathways are stimulated and platelet activation is triggered resulting in secretion of the α and dense (δ) granule contents. The extent to which platelets release their granular cargo depends on the strength and type of stimulus. Whereas large molecules such as fibrinogen, factor V, P-selectin and multiple chemo- and cytokines are stored in the α granules, the dense granules contain amongst others ATP, ADP and polyphosphates. Along with thromboxane A2 (TXA2) generated by the cyclooxygenase/thromboxane synthase enzymes, ATP and ADP serve as important autocoids that upon release activate nearby platelets and as such promote the incorporation of more platelets from the circulation into the growing platelet plug. ATP signals via the purinergic receptor P2X1, while ADP activates P2Y1 and P2Y12. Within the growing platelet plug, platelet aggregation is dependent on integrin $\alpha_{IIb}\beta_3$, a glycoprotein abundantly expressed on the platelet surface. Upon platelet activation, integrin $\alpha_{IIb}\beta_3$ changes its confirmation and is thereby able to bind fibrinogen and to support platelet-platelet interactions. Concurrently, thrombin generated by the coagulation cascade additionally enhances platelet activation through activation of the PAR-1 and PAR-4 receptors present on the platelet membrane. Upon strong activation with sustained high intracellular Ca^{2+} levels, platelets become procoagulant. By exposing phosphatidyl serine on their outer leaflet of the plasma membrane, this subpopulation of procoagulant platelets provides a negatively charged surface for the assembly of the tenase and prothrombinase complexes and thus supports coagulation. Further crosstalk between the coagulation cascade and platelets occurs upon the formation of fibrin as fibrin can interact with the GPVI receptor on platelets, enabling platelets to bind to fibrin within the clot.

Different types of atherothrombosis

In accordance with Virchov's triad, thrombosis typically involves three elements, including blood flow, blood composition and vessel wall. The weight of each Virchov element may vary depending on the underlying disease: Commonly, an interaction between blood and the damaged atherosclerotic vessel wall triggers arterial thrombosis (Figure 50.1c).

Already in the early stages of atherosclerotic plaque development endothelial cell dysfunction occurs; platelets adhere to the dysfunctional endothelium and as such are able to deposit their granular cargo and release platelet microparticles. They thereby add to the endothelial cell dysfunction, mediated by amongst others CCL5/RANTES, CXCL4/platelet factor 4 and IL-1 and contribute to plaque development. Subsequently, plaque development gradually progresses and more pro-inflammatory and prothrombotic properties accumulate, until in the final stages of atherosclerosis, plaque rupture or erosion occurs. The resulting exposure of highly thrombogenic plaque material to the circulation triggers the formation of (occlusive) thrombi. Interestingly, plaque erosion and plaque rupture are associated with the formation of distinct types of thrombi. Whereas occlusive, luminal thrombi that are exposed to the necrotic core can be found on plaques that have ruptured, thrombi that are mural and non-occlusive typically form on eroded plaques. Furthermore, clot composition is different. Ruptured plaques support the formation of fibrin-rich thrombi (fibrin area: 75%, platelet area: 35% in thrombi), whereas thrombi formed on eroded plaques are rich in platelets (platelet area: 70%, fibrin area: 51% in thrombi). These thrombi enriched in platelets appear to be more prevalent in patients with non-ST segment elevation myocardial infarction (NSTEMI), while fibrin, erythrocyte-rich thrombi are more frequent in patients with acute myocardial infarction (MI). At this stage, these apparent histological differences do not yet translate into specific antithrombotic strategies for the different subtypes of MI.

Antithrombotic treatment for primary prevention of atherothrombosis

Single antiplatelet agents: mode of action, side effects

Aspirin is the common name for acetylsalicylic acid, a compound that acts by inhibiting prostaglandin synthesis in different cells; the relevant antithrombotic effect is thought to be mostly based on inhibition of the cyclooxygenase-1 production of TXA2 in platelets (Figure 50.2). It is prescribed at doses between ±75 and 325 mg od, mostly dependent on regional differences (US more commonly higher doses than Europe). Its intake results in irreversible inhibition of platelet activation and aggregation. To achieve sustained effect, daily intake of aspirin is needed to suppress newly formed platelets, about 10% per day.

Clopidogrel is a prodrug that needs metabolisation in the liver. It is ingested as a single dose of 75 mg/day and the active metabolite shows a large interindividual variation in blood. This variability is partly explained by genetic polymorphisms encoding cytochrome P450 (CYP) 2C19, the hepatic enzyme involved in biotransformation of the prodrug clopidogrel to its active metabolite. The active metabolite interferes with the binding of ADP to the platelet P2Y12 receptor. This

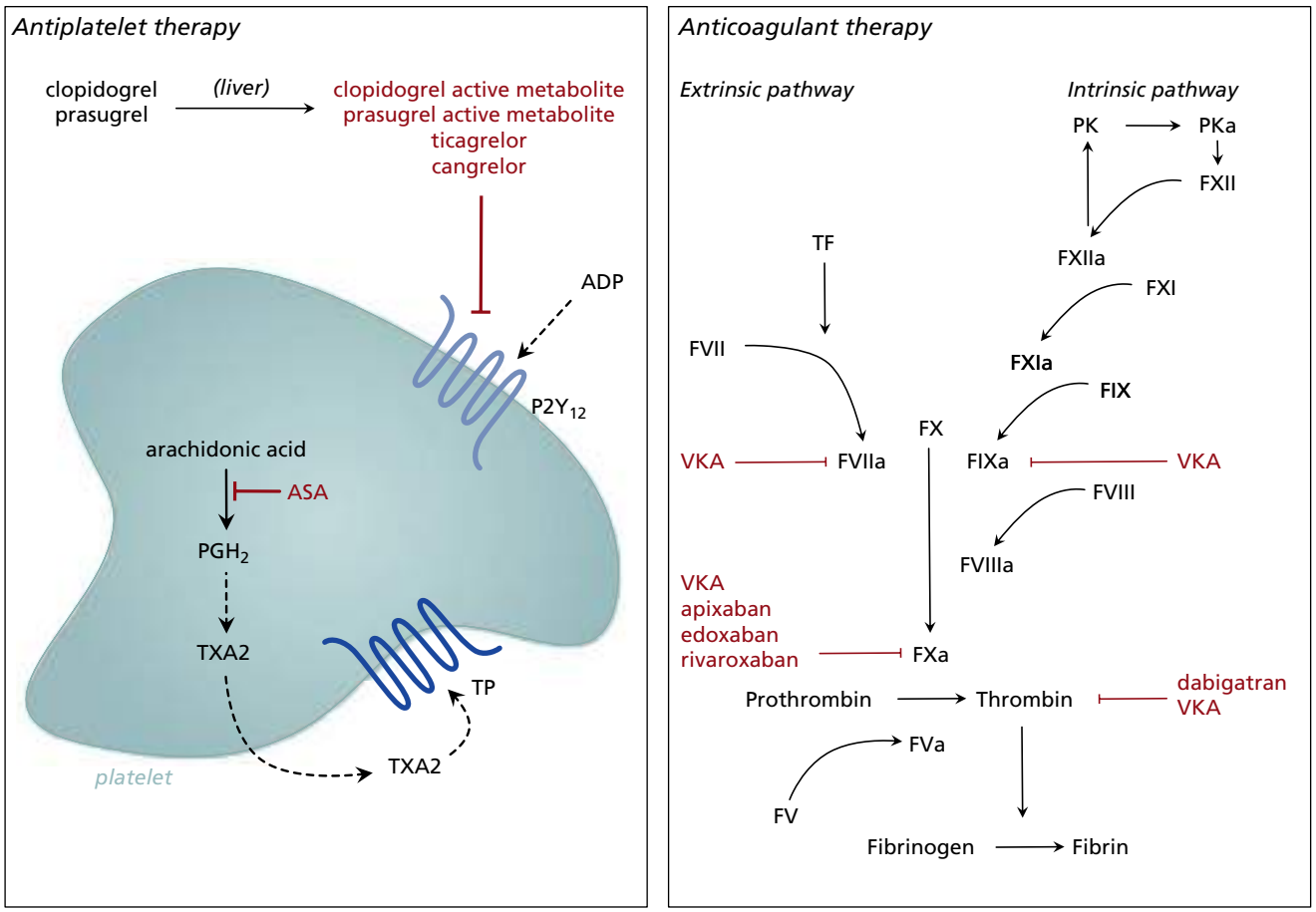

Figure 50.2 Left panel shows sites of interaction and mechanistic pathways involved in the action of aspirin and P2Y12 inhibitors; right panel illustrates the mode of action of current anticoagulants, with vitamin K antagonists (VKA) targeting the carboxylation of vitamin K-dependent proteins by the liver and direct oral anticoagulants (DOAC), directly targeting activated factor X or thrombin.

pharmacodynamic variation translates into variation in clopidogrel effectiveness after percutaneous coronary intervention (PCI). The interindividual variation in response to clopidogrel, inappropriately referred to as clopidogrel "resistance," was first identified in patients with coronary disease, occurring up to 25% of cases in platelet function testing (depending on the test used). These patients have less active metabolite and high on-treatment platelet reactivity (HTPR) while on clopidogrel; this is associated with increased incidence of major adverse cardiovascular events (MACE). Several studies addressed the question of whether clopidogrel dose adjustment based on platelet function testing, would correct this problem, but the outcome of such studies was consistently negative. However, more recent studies showed that when clopidogrel is prescribed based on the genetic profile of *CYP2C9* metabolism (translating into poor, intermediate, good or strong metabolisers), it is possible to achieve appropriate efficacy with this drug, in high-risk coronary patients (see further).

Another option is to use more potent P2Y12 inhibitors, which are either not hindered by this CYP2C9 metabolism such as prasugrel, or are not pro-drugs (ticagrelor) and therefore have a more predictable pharmacodynamic profile. Clopidogrel and prasugrel are irreversible platelet inhibitors, while ticagrelor binds reversibly.

The principal side effect of aspirin is bleeding and the regular use of aspirin increases the risk of gastro-intestinal bleeding twofold. Risk factors for bleeding should therefore be taken into account, including (recent) gastrointestinal (GI) ulcer (H. pylori infection may be a modifiable risk factor), old age, use of interacting medication including other antiplatelet therapy (APT), non steroidal anti-inflammatory drugs (NSAIDs), COX-2 selective inhibitors, oral anticoagulants, corticosteroids. In general, the risk of GI bleeding with aspirin can be effectively reduced with proton pump inhibitors (PPI), better than with histamine-2 receptor antagonists. The introduction of a PPI increases costs and also has potentially relevant side effects in the long run. Alternative options may be phospholipid-coated

aspirin preparations that in contrast to enteric-coated aspirin, have equivalent efficacy for inhibiting TXA2 formation as compared to aspirin.

Although clopidogrel does not interfere with prostaglandins in gastric mucosal tissue, its use is also associated with an increased risk of bleeding, of which GI bleeds are the most common type. The benefit of standard use of a PPI in patients on clopidogrel has been disputed, in fact, there is evidence that its concomitant use is associated with an increased risk of MACE. Compared to clopidogrel, the newer and more potent P2Y12 inhibitors prasugrel and ticagrelor are associated with a further increase in the risk of major bleeding (see also Section "Antithrombotic therapy in patients with acute coronary syndrome"). These agents are generally not prescribed as single APT, although ticagrelor may be used as a single agent in selected patients. Although the thienopyridine prasugrel is a prodrug like clopidogrel, it only requires a single oxidation step to form its active metabolite, and it seems to be not affected by genetic variations in CYP enzymes. Compared to clopidogrel, its use is associated with an increase in the risk of major bleeding, especially amongst those with age ≥75 years or body weight <60 kg such that its use is contraindicated in patients with prior stroke. Ticagrelor, a reversible P2Y12 receptor antagonist, provides faster and stronger platelet inhibition with less patient-to-patient variation. In most instances, ticagrelor and prasugrel will be prescribed as part of dual APT (DAPT) to be discussed in later sections. In order to reduce the risk of GI bleeding while on DAPT, PPI's will be additionally prescribed in most cases.

Primary prevention in subjects without apparent CVD

Given the efficacy/safety profile and the low costs, *primary* prevention with aspirin has been extensively studied. Recent studies and meta-analyses of large trials refute a major role for aspirin in primary prevention. In subjects with a specific risk factor like diabetes, aspirin can be considered in those with ≥1 organ damage or ≥3 major risk factors, or any risk factor and ≥10-year disease duration without organ damage, based on a meta-analysis of 12 randomised controlled trials, including the recent ASCEND trial that showed that aspirin reduced MACE by 11% compared with placebo. In conditions with high platelet turnover, twice daily administration of aspirin may be more effective and can be considered in specific conditions, such as subjects with myeloproliferative disease and high platelet production and turnover.

Primary prevention in subjects with documented atherosclerosis; general considerations

Primary lifelong prevention (of MACE) with aspirin or clopidogrel is indicated in all persons with *symptomatic* atherosclerotic disease, including angina and symptomatic PAD, as the benefits outweigh the bleeding risk. The benefit of aspirin has not yet been established for subjects with *asymptomatic* lower extremity artery disease (LEAD), although mortality is comparable to patients with symptomatic disease. In patients with symptomatic PAD, clopidogrel is preferred over aspirin based on the PAD subgroup analysis of the CAPRIE trial showing superior antithrombotic efficacy of clopidogrel at comparable bleeding risk. However, both aspirin and clopidogrel are still used for this indication.

Single APT, either with aspirin or clopidogrel, is also recommended in carotid artery disease with carotid artery stenosis >50%.

Stronger acting APT like ticagrelor may be used as alternative in patients with PAD in case of failure, allergy or "resistance" to aspirin or clopidogrel, based on the Euclid trial showing non-inferiority of ticagrelor versus clopidogrel.

Since the residual risk of atherothrombotic complications including mortality remains at around 10% annually in PAD patients, more intensive antiplatelet regimens have been assessed as well. Importantly, the efficacy benefits of DAPT were first established in a population with coronary artery disease in the PCI-CURE study (DAPT is further discussed in section "Antithrombotic therapy in patients with acute coronary syndrome"). Assuming similar benefits could be achieved in other high-risk for thrombosis populations, DAPT was tested in large trials like CHARISMA but with negative outcomes; no gain in efficacy and more bleeding. Consequently, in patients with non-acute, symptomatic atherosclerotic disease, DAPT with aspirin and clopidogrel is only recommended following surgical intervention of the carotid arteries (at least 1 month), or after percutaneous intervention in peripheral arteries of the legs for 1 month, followed by single APT.

The use of oral anticoagulants (mostly vitamin K antagonists) in therapeutic intensity is not indicated in patients with PAD, except for those who undergo venous bypass grafting. The most recent regimen studied in the COMPASS study involving patients with high-risk arterial vascular disease, including PAD, is the combination of the anticoagulant rivaroxaban 2.5 mg bd (Figure 50.2), and low-dose aspirin, which reduced cardiovascular mortality as well as major acute limb events in patients with PAD (patients with LEAD or carotid artery disease).

Primary prevention in patients with stable, symptomatic coronary artery disease

All patients with symptomatic coronary artery disease require APT and, in most cases, aspirin will be prescribed lifelong; clopidogrel is an alternative option. Aspirin is prescribed in a relatively low dose of 75–100 mg, either as acetylsalicylic acid or as calcium-carbasalate, although in the USA higher doses of 325 mg/day of acetylsalicylic acid may be used. Pharmacologically, there is not

much evidence for a benefit of higher doses of aspirin as compared to low doses of 75–100 mg od.

Many patients with angina will at some stage undergo vascular imaging to determine the cause of complaints and in case of significant atherosclerotic lesions, PCI and stenting is a next management step. Upon stenting, these patients will be treated with DAPT for a period of usually up to 6 months, depending on estimated risks of thrombosis and bleeding. Hence, in case of a bleeding risk, duration of DAPT after elective PCI in patients with chronic CAD may be limited to 1–3 months. Obviously, risk factors for bleeding should always be addressed and if possible managed. Alternatively, in those at high risk of recurrent thrombosis, continued DAPT may be considered; however, this policy has no favourable effect on mortality while bleeding risk doubles as compared to single APT. Combining conventional oral anticoagulants with APT in patients with stable CAD (and without AF) is not cost effective, but low-dose rivaroxaban (2.5 mg bd) plus aspirin may provide a useful strategy in particular CAD patients with an estimated high risk of thrombosis.

For the long term, in high risk for thrombosis CAD patients, the choice of single APT, or DAPT, or rivaroxaban plus aspirin (dual pathway inhibition) must be carefully balanced. A network meta-analysis of randomised controlled trials suggests an efficacy benefit for any combination with aspirin at the price of an increased bleeding risk. Taking into account outcomes of all ischaemic and bleeding events and all-cause mortality, rivaroxaban plus aspirin appeared to be the preferred long-term antithrombotic regimen for patients with chronic coronary syndrome and high-risk factors.

In patients with CAD requiring coronary artery bypass grafting (CABG), aspirin will be continued during surgery (supported with systemic heparinisation during the extracorporeal bypass procedure). Following surgery, the patient will usually continue with single APT, lifelong, because of underlying atherosclerosis.

Antithrombotic therapy in patients with acute coronary syndrome

Aspirin (bolus 150–300 mg orally or 75–250 mg i.v. loading dose, followed by low dose 75–100 mg QD) is indicated as soon as possible in all forms of acute coronary syndrome (ACS) based on the ISIS-2 trial. Aspirin halves the risk of recurrent myocardial infarction (MI, 1.0 versus 2.0%) and reduces vascular mortality (Figure 50.3). The downside of aspirin treatment is an increased bleeding risk, which is predominantly gastro-intestinal of origin.

Parenteral anticoagulation is also indicated in the acute phase of all forms of ACS. Unfractionated heparin (UFH) or the low molecular weight heparin (LMWH) enoxaparin is indicated in STEMI, while the factor Xa-inhibitor fondaparinux is not indicated due to more thrombotic complications in patients

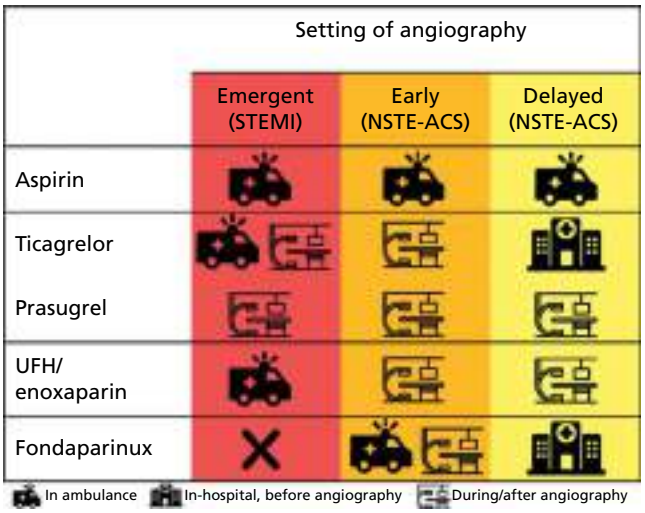

Figure 50.3 Timely use of antithrombotic therapy in the acute phase of ACSACS: acute coronary syndrome; STEMI: ST-segment elevation myocardial infarction; NSTE-ACS: non-ST-elevation ACS i.e. non-ST-elevation myocardial infarction or unstable angina pectoris; UFH: unfractionated heparin.

undergoing primary PCI. In NSTEMI the preferred agent is UFH when an early invasive strategy is chosen or fondaparinux when a conservative or delayed invasive strategy is preferred. Even when the patient is treated with fondaparinux, during coronary angiography and PCI, additional UFH is indicated as fondaparinux does not prevent catheter thrombosis, which may inflict myocardial infarction. In ACS (STEMI and NSTEMI), it is advised to administer anticoagulation until PCI or for 5 days or until discharge.

In addition to aspirin, P2Y12 inhibition is indicated in ACS. In all ACS patients, the stronger – and faster-acting agents ticagrelor or prasugrel are preferred over clopidogrel as they demonstrated superiority in the pivotal trials TRITON and PLATO, together containing over 30,000 patients. However, both ticagrelor and prasugrel increase bleeding risk, and this risk has been shown to be associated with increased morbidity and mortality for more severe bleeding.

As the thrombotic risk in ACS is highest in the early hours, it is preferred to load the patient with STEMI with one of the stronger agents, while de-escalation in-hospital is an option in those patients with a high bleeding risk. There is still controversy about when to load the STEMI patient with oral P2Y12 inhibition, whether this is in the ambulance or after coronary angiography when the coronary anatomy is known. Benefit from in-ambulance administration may be limited by slow onset of action of oral P2Y12 inhibition (2–4 hours) through gastro-intestinal absorption, even more, delayed when morphine is co-administered. Several studies found faster platelet inhibition with chewed or crushed rather than integral administered P2Y12 inhibitors; however in the COMPARE

CRUSH trial, this was not translated into improved coronary flow or ST-segment resolution.

Another option to reach rapid platelet inhibition in the early phase of STEMI is the use of intravenous platelet inhibitors. Glycoprotein IIb/IIIa inhibitors have been shown to improve pre-pPCI target vessel patency, post-pPCI ST-segment resolution and patient outcomes. However, because of slow offset of action, bleeding risk and thrombocytopenia and introduction of the more potent P2Y12 inhibitors for these patients, they are mainly used for bail-out purposes in case of suboptimal stenting or high thrombus load. Another intravenous agent, the P2Y12 inhibitor cangrelor, which has a fast on- and off-set, might also be considered for patients who have no or suboptimal P2Y12 inhibition when PCI is performed. It reduces thrombotic complications 48 hours after PCI, 3.8 versus 4.7% compared with clopidogrel (odds ratio [OR] 0.81 [95% CI 0.71–0.91]); but also comes with increased mild bleeding complications in around 6% of cases.

In NSTEMI, the choice and timing of P2Y12 inhibition depend on the preferred management (invasive or conservative) and, if coronary angiography is preferred, when this is performed (early or delayed). Loading with P2Y12 inhibitors before coronary anatomy (thus before coronary angiography) is known, is referred to as pre-treatment. Pre-treatment with half-dose prasugrel did not reduce thrombotic events but increased major bleeding in the ACCOAST trial, where all patients underwent early coronary angiography. In the ISAR REACT 5 trial, prasugrel started after coronary angiography was superior to ticagrelor before angiography in ACS patients. Bleeding events did not differ between groups. Based on the ACCOAST and ISAR-REACT 5 trials, the ESC guidelines advise, if an early invasive strategy is preferred, to wait with loading until the coronary anatomy is known and the patient is to proceed to PCI. In patients in whom a conservative or delayed invasive strategy is preferred, pre-treatment remains an option in patients with a higher thrombotic risk and ticagrelor is preferred unless the patient has a higher bleeding risk, then clopidogrel is a safer option. In patients treated conservatively, the ESC guidelines advise to use ticagrelor, while prasugrel is contraindicated. In patients with a high bleeding risk especially in the elderly, clopidogrel may be a better option.

Antithrombotic treatment at discharge

Most patients with ACS undergo PCI with stenting. In these patients, DAPT is essential to prevent ST but also spontaneous MI and stroke. When determining the most suitable P2Y12 inhibitor as well as the duration of DAPT, the associated bleeding risk has to be balanced against the reduced thrombotic risk. The 12-month duration of DAPT in ACS is based on the TRITON and PLATO trials. Since then, the newer generations drug-eluting stents (DES) that are used and also better implantation techniques have led to lower rates of stent thrombosis, challenging the need for long duration of DAPT.

Several RCTs tested a shortened (1–6 months as compared to 12 months) duration of DAPT. All of these trials have shown reduced bleeding, while only a few demonstrated a higher thrombotic risk with a shorter DAPT duration. Based on the results of these trials, in line with the ESC guidelines, we consider 1-year DAPT to be recommended in ACS. However, a shorter course could be considered in patients at higher risk of bleeding complications.

When DAPT is discontinued, patients are usually treated with lifelong aspirin monotherapy to prevent recurrent atherothrombotic events. A recent study, the HOST EXAM trial, randomised Korean patients in their chronic maintenance period after PCI to clopidogrel or aspirin monotherapy. It showed that clopidogrel might be a favourable option both with regards to thrombotic (3.7 versus 5.5%, HR 0.68 [95% CI 0.52−0.87]) and bleeding complications (2.3 versus 3.3%, HR 0.70 [95% CI 0.51–0.98]). Several trials had already tested P2Y12 inhibitor monotherapy after 1–3 months of uneventful standard DAPT, similar to the trials studying a shortened DAPT as described above. In general, these trials found P2Y12 inhibitor monotherapy (mostly ticagrelor) equally effective as compared to standard DAPT, whilst reducing bleeding (but not major bleeding) complications. Based on these results, P2Y12-inhibitor monotherapy after an initial short course of DAPT should be an alternative for standard DAPT in patients undergoing PCI without high ischaemic risk. Based on the HOST EXAM trial, also clopidogrel might be preferred over aspirin after one-year DAPT post-ACS.

Risk scores or criteria such as PRECISE-DAPT and ARC-HBR may aid decision-making. They should be applied at the time of PCI to guide DAPT duration. The PRECISE-DAPT score is a five-item risk score to predict out-of-hospital bleeding after PCI. Amongst patients with a high bleeding risk (PRECISE-DAPT score $\geq$25), standard DAPT was associated with no reduction in ischaemic events, but a strong increase in bleeding with a number needed to harm of 38. In contrast, standard treatment in patients without high bleeding risk (PRECISE-DAPT score <25) was associated with significant reductions in the combined ischaemic endpoint, without an increase in bleeding, with a number needed to treat of 65. Thus, by upfront deciding on a short course of DAPT in patients at high bleeding risk, a substantial proportion of excess bleeding events might be prevented, while patients without high bleeding risk benefit from standard or extended DAPT. Noteworthy, in five out of eight RCTs included in the PRECISE-DAPT derivation cohort, exclusion criteria such as thrombocytopenia, anaemia or history of bleeding were applied. In the derivation cohort, the incidence of bleeding was relatively low (1.5 and 0.8% for major and minor and major bleeding events, respectively) suggesting that patients at highest risk of bleeding were not included. The expert consensus-derived ARC-HBR criteria are relatively new and not extensively studied yet. It should be noted that

risk-score-guided DAPT duration has never been compared to not risk-score guided decision-making in a randomised fashion.

Antithrombotic therapy in ACS patients with concomitant indication for OAC

A special population of interest is patients that have a concomitant indication for oral anticoagulants (OAC; mostly atrial fibrillation), since combination therapy with antiplatelet therapy increases bleeding risk significantly. It is known from the AFIRE and the OAC-ALONE trials, that in stable CAD combination therapy of OAC and antiplatelet therapy should be avoided for its clear harm with regards to bleeding complications, without antithrombotic benefit. The occurrence of an ACS however, demands temporary use of APT to prevent recurrent MI and ST. Combination therapy of OAC, P2Y12 inhibitor and aspirin is referred to as triple therapy, whereas combination therapy with OAC and P2Y12 inhibitor without aspirin is referred to as dual therapy (in contrast to dual antiplatelet therapy [DAPT]). The pivotal WOEST trial was the first to show that, omitting aspirin (thus dual therapy) reduced bleeding from 44.4 to 19.4% (HR 0.36, 95% CI 0.26–0.50) without increased MACE. The PIONEER, RE-DUAL, ENTRUST and AUGUSTUS trials confirmed this. The AUGUSTUS trial showed in addition, that DOAC should be preferred over VKA, both for bleeding reduction and antithrombotic efficacy (patients with mechanical heart valve prosthesis or other contra-indication to DOAC were not included). Post-hoc analysis also found, that if aspirin was used, its benefit was only observed in the first 30 days after PCI. Therefore, in patients with a high thrombotic risk, aspirin could be used but its duration should be limited to the in-hospital phase, with extension to 30 days only in rare high-thrombotic risk cases. Duration of P2Y12 inhibition has not been the subject of study in patients with dual therapy and should be similar to those without OAC. The choice of P2Y12 inhibitor is usually clopidogrel for its more favourable bleeding risk as compared to ticagrelor or prasugrel, however not backed by sound evidence with regards to antithrombotic efficacy.

Guided and non-guided de-escalation

Switching from the potent P2Y12 inhibitors ticagrelor or prasugrel to the weaker clopidogrel for its more favourable bleeding profile is referred to as de-escalation. De-escalation can be guided or unguided. Unguided de-escalation is initiated after a certain period of time in a population at higher bleeding risk such as the elderly. Guided de-escalation can be based on platelet function testing or genetic profile and is usually initiated soon after PCI; current guidelines advice this strategy for selected patients ineligible for 12 months of potent P2Y12 inhibition.

Unguided de-escalation

Two trials have investigated unguided de-escalation from ticagrelor or prasugrel to clopidogrel after one month of uncomplicated DAPT. The TOPIC and TALOS-AMI trials both found de-escalation to reduce bleeding (4.0 versus 14.9%, HR 0.30 [95% CI 0.18–0.50]; and 3.0 versus 5.6%, HR 0.52 [95% CI 0.35–0.77], respectively) without an increase in thrombotic events or MACE at 12 months. The HOST-REDUCE-POLYTECH-ACS randomised ACS patients after one month to half or full dose prasugrel in a Korean population. The reduced dose was associated with less bleeding (2.9 versus 5.9%, HR 0.48 [95% CI 0.32–0.73]), and no increase in efficacy events. Finally, the POPular AGE trial randomised patients aged ≥70 years of age about one day after ACS to clopidogrel or ticagrelor. The use of clopidogrel reduced bleeding complications (18 versus 24%, HR 0.71 [0.54–0.94]) without difference in MACE. Thus, unguided de-escalation after one month to clopidogrel or half-dose prasugrel reduces bleeding and seems as effective as maintained potent P2Y12 inhibition. Immediate unguided de-escalation has been studied in elderly and may also be an option for selected patients.

Platelet function guided de-escalation

The TROPICAL ACS trial investigated platelet function-guided de-escalation from prasugrel to clopidogrel after 1 week of prasugrel treatment. The investigational study arm was treated with clopidogrel for 1 week, and patients then demonstrating high-platelet reactivity switched back to prasugrel, while the patients with normal platelet reactivity remained on clopidogrel until 12 months after MI. Patients in the control arm were treated with prasugrel for 12 months. The platelet function-guided strategy did not significantly reduce bleeding complications, nor MACE. However, as this strategy poses no benefit with regard to bleeding reduction, its value may be less clear than other de-escalation strategies.

Genotype guided de-escalation

The POPular Genetics trial investigated genotype-guided P2Y12 inhibitor de-escalation in STEMI patients. As clopidogrel is a prodrug that becomes active through CYP2C19 which has several common loss-of-function alleles, it might be appropriate to prescribe the generally weaker clopidogrel if absence of these alleles can be confirmed. The study randomised patients 1–3 days after STEMI to genotype-guided clopidogrel (61% of the population) or to potent P2Y12 inhibitor in carriers of a loss-of-function allele. The control group consisted of potent P2Y12 inhibition. The genotype strategy significantly reduced bleeding complications (9.8 versus 12.5%, HR 0.78 [95% CI 0.61–0.98]) whilst maintaining similar antithrombotic efficacy (MACE 2.7 versus 3.3%, HR 0.83 [95% CI 0.53–1.31). Thus, after ascertaining the pharmacogenomic effectiveness of

clopidogrel administration, this may be a feasible option in ACS patients as well, with less bleeding complications as compared to unguided prescription of potent P2Y12 inhibitors.

As stated above, all of the above options may be considered for selected patients for whom (maintained) potent P2Y12 inhibition is undesirable.

Residual risk at 1 year after acute coronary syndrome

While patients with a higher bleeding risk benefit from a reduced duration of DAPT, patients with a higher thrombotic risk may benefit from an extended (>1 year) duration of DAPT (Figure 50.4). When balancing the risks and benefits of extended DAPT, we have to consider that the overall thrombotic risk – including mortality – after ACS has decreased due to evidence-based treatments and the use of newer generation DES leading to decreased rates of especially late ST. Therefore, DAPT late after ACS to reduce ST is not needed in the majority of patients. On the other hand, DAPT also reduces spontaneous MI, stroke, and in the highest-risk patients, mortality but at the cost of more bleeding. Further, it is important to acknowledge the different grades of severity in both bleeding and ischaemic events, as not all events have the same impact on mortality. Therefore, a careful assessment should be made to select those patients who may benefit from extended antithrombotic therapy.

The DAPT study showed superiority of continued 30 months DAPT compared to standard 12 months of DAPT in the occurrence of MI and ST, while moderate or severe bleeding was increased to a similar degree. Importantly, non-cardiovascular death was numerically higher, suggesting that not all patients should be treated with DAPT beyond 12 months. This topic was further studied in the PEGASUS-TIMI 54 trial, including only patients who had had a previous MI and had additional thrombotic risk factors. At 3 years, DAPT (aspirin plus ticagrelor 90 and 60 mg BID) reduced the composite of cardiovascular death, MI or stroke. As expected, major bleeding was higher with ticagrelor again roughly in a similar degree as the reduction of thrombotic events. Therefore, these data suggest that only patients who have had a spontaneous ACS are candidates for extended DAPT, which is in line with the latest ESC guidelines recommendations: extended DAPT should be considered in ACS patients at high ischaemic risk without increased risk for major or life-threatening bleeding (class IIa, level of evidence A).

Another option to reduce the long-term thrombotic risk in ACS patients may be dual pathway inhibition (rivaroxaban

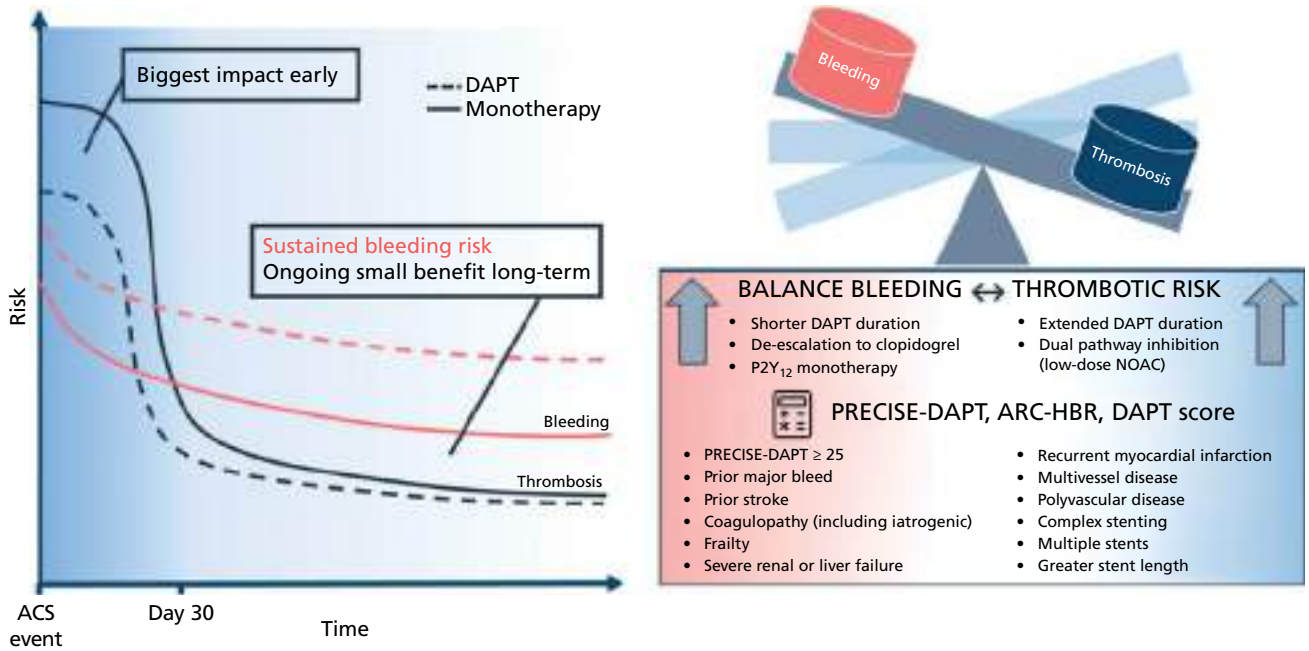

Figure 50.4 Optimal duration of dual antiplatelet therapy (DAPT) DAPT decreases thrombotic risk after acute coronary syndromes (ACS) whereas it increases bleeding risk. Its benefit is greatest in the early phase and diminishes but is sustained on the long term. Personalised decisions should be made regarding earlier de-escalation for those at high bleeding risk, and prolonged or intensified treatment for those at high thrombotic risk in absence of bleeding complications. Risk scores and clinical risk factors for bleeding or thrombotic events can guide treatment decisions.

[2.5 mg BID] on top of aspirin), which reduces the incidence of cardiovascular death, MI and stroke in the ATLAS ACS 2-TIMI 51 trial. The COMPASS trial also tested dual pathway inhibition and included CCS patients of which >50% had a previous MI. The composite of cardiovascular death, stroke or MI was lower with rivaroxaban (2.5 mg BID) plus aspirin than with aspirin alone. Major bleeding, but not fatal bleeding, was higher with rivaroxaban plus aspirin than with aspirin alone. Importantly, death occurred in 3.4% with rivaroxaban plus aspirin as compared with 4.1% with aspirin alone (HR 0.82, 95% CI 0.71–0.96). Risk reductions with rivaroxaban plus aspirin were larger in patients at high ischaemic risk (e.g. both coronary artery disease and peripheral artery disease or concomitant diabetes). Also, the 2020 ESC guidelines for the management of ACS patients without persistent ST-segment elevation state that rivaroxaban 2.5 mg BID plus aspirin should be considered in patients at high thrombotic risk and without an increased risk for major or life-threatening bleeding.

So, although there is evidence for extended antithrombotic therapy in ACS patients, there is no consensus on which patients this can be applied. This is due to the fact that 'higher thrombotic risk' is not well defined and that many patients with a higher thrombotic risk also have a higher bleeding risk. In addition, there is also no consensus in whom to continue DAPT and in whom to use dual pathway inhibition. In deciding on optimal antithrombotic therapy, risk stratification is essential and according to the ESC guidelines. The PRECISE-DAPT or ARC-HBR score could be used at discharge to reduce the duration of DAPT in those patients with a higher bleeding risk. Further, residual thrombotic risk at 1 year should be assessed in all patients that tolerated DAPT and had no thrombotic or bleeding event. To select patients that may benefit from extended DAPT, the ESC guidelines advise the use of the DAPT score, the criteria used in the PEGASUS-TIMI 54 study, or the criteria for high ischaemic risk mentioned in the ESC guidelines, which consist of clinical and angiographical/procedural risk factors (i.e. diabetes mellitus requiring medication, polyvascular disease, bifurcation stenting, etc.). It is noteworthy that the ESC criteria have not yet been extensively validated, and that none of these risk scores has been tested in a randomised fashion against no use of a score. Finally, extended DAPT and dual pathway inhibition with aspirin plus low-dose rivaroxaban have not been compared head-to-head to extended DAPT and therefore make the choice for the best antithrombotic therapy difficult. An option may be to keep the patient on DAPT who has well-tolerated DAPT and start low-dose rivaroxaban in a patient with a high thrombotic risk treated with aspirin alone especially when this patient has both coronary artery and peripheral arterial disease. When DAPT is continued, ticagrelor 60 mg BID is preferred, but there is also data for both clopidogrel and prasugrel from the DAPT trial. The data supports the use of extended antithrombotic therapy up to 4 years post-ACS, but some patients at a continuous high ischaemic risk may have a lifelong indication as long as the bleeding risk is not increased. A yearly evaluation of both the thrombotic and the bleeding risk is therefore mandated.

Secondary prevention in transient ischaemic attack and ischaemic stroke (non-embolic)

TIA and minor ischaemic stroke account for 30–50% of all cerebral ischaemic events. Aspirin with or without dipyridamole has been the mainstay of antithrombotic management for decades. In the large PRoFESS, trial aspirin with dipyridamole was compared with clopidogrel showing no difference in efficacy, but with fewer gastrointestinal bleedings in the clopidogrel arm, leading to a further increment in use of clopidogrel only for secondary stroke prevention.

In patients with acute ischaemic stroke or TIA, the combined treatment of aspirin and clopidogrel is recommended for 21 days; longer use of DAPT has no benefit due to increased bleeding risk. A major disadvantage of clopidogrel, as discussed before, is its variable and genetically determined metabolism, resulting in variable concentrations of active metabolite, hence a variable degree of inhibition of the platelet P2Y12 inhibitor. Current studies suggest that the presence of poor metabolism status (genotyped) associated with HTPR (platelet phenotyping) for clopidogrel, is a risk factor for recurrent stroke, as well as for worse stroke outcome. Platelet reactivity while on clopidogrel does not seem to be associated with bleeding risk. Such data suggest that at least genotyping patients with stroke for sensitivity to clopidogrel would be helpful in selecting this agent for long-term secondary prevention, or not.

Given the variable degree of platelet inhibition of clopidogrel, different strategies have been tested in the management of acute ischaemic stroke or TIA, including aspirin plus clopidogrel versus aspirin alone (CHANCE trial) showing superiority for DAPT over the initial 3 weeks. Assuming that a more potent P2Y12 inhibitor would have advantage over aspirin and clopidogrel, the SOCRATES study tested ticagrelor versus aspirin in acute TIA/minor stroke; ticagrelor was not superior over aspirin except for a pre specified subgroup of patients with >50% stenosis of the ipsilateral carotid artery. The subsequent THALES trial tested the combination of ticagrelor plus aspirin versus aspirin alone in high risk for recurrent stroke patients, showing superiority with regard to reducing recurrent stroke or death, but not death alone by 30 days. The price was a fourfold increase in major, but not fatal bleeding at 5 days already.

Studies comparing aspirin plus clopidogrel versus aspirin plus ticagrelor may be needed to assess the relative efficacy and safety of each DAPT combination for the initial management of acute stroke or TIA, for 3 weeks. Whether DAPT is also an option for those with clopidogrel resistance is subject of ongoing study. Whether other combined antithrombotic strategies

including aspirin or clopidogrel in combination with cilostazol will be of benefit in high risk for recurrent stroke patients, remains to be studied. Prasugrel, another potent P2Y12 inhibitor is contra-indicated in stroke patients, for increased risk of intracranial bleeding. Dual pathway inhibition with rivaroxaban and aspirin cannot be applied in these patients due to lack of evidence (these patients were not recruited in the Compass trial).

Arterial thrombosis in the heart; antithrombotic management in patients with AF

Thrombus formation in the heart can have different origins, depending on underlying disease and localisation. In case of AF, thrombus formation occurs in the left atrium and in particular the left atrial appendix (Figure 50.5). In AF, all elements of Virchov's triad are affected; flow conditions are obviously altered and contribute to abnormal patterns of blood circulation, increasing interaction of blood cells with the atrial endothelium. Within the left atrium and appendix orifice, the endothelial phenotype is altered to stimulate blood coagulation, while fibrinolysis is impaired due to an imbalance between local tPA and PAI-1 concentrations. In conjunction with advanced age and comorbidity (e.g. hypertension) inflammatory mechanisms will further perturb the endothelium towards a prothrombotic surface. Systemically, elevated markers of platelet activation suggest platelet hyperreactivity and systemic hypercoagulability is a key element of AF too, already detectable at an early stage in otherwise apparently healthy subjects (lone AF). At advanced age, thrombo-inflammatory effects will further increase, which explains the increased risk of thromboembolic stroke. The interplay of elements is apparently such, that this process is poorly inhibited with antiplatelet agents as shown in clinical trials. Hence, different from atherosclerosis, the endothelial cell properties in concert with flow and hypercoagulability conditions, favour a clotting process that is best inhibited with anticoagulants.

AF is a common disorder, occurring in an age-dependent manner with marked increase in prevalence over the age of 70 years, both in men and women. The risk of thromboembolism in AF is mostly estimated by the CHA_2DS_2-VASc (Congestive heart failure, Hypertension, Age ≥75 years, Diabetes mellitus, Stroke, Vascular disease, Age 65–74 years, Sex category [female]) score (Table 50.1). This score is central in risk classification according to current ESC guidelines, taking into account its limitations mainly involving fairly gross risk estimations. Further refinement may be possible by also including certain biomarkers as in the ABC (Age, Biomarker, Clinical history) risk score. A Patient-Centred Outcomes Research Institute (PCORI)-commissioned systematic review of 61 studies comparing diagnostic accuracy and impact on clinical decision making concluded CHA_2DS_2-VASc and ABC risk scores to have the best evidence for predicting thrombo-embolic risk. In all scores, the total number of points guides the decision to start with oral anticoagulant therapy (Table 50.1).

The decision to actually prescribe anticoagulation is also influenced by considering bleeding risk-enhancing factors, most

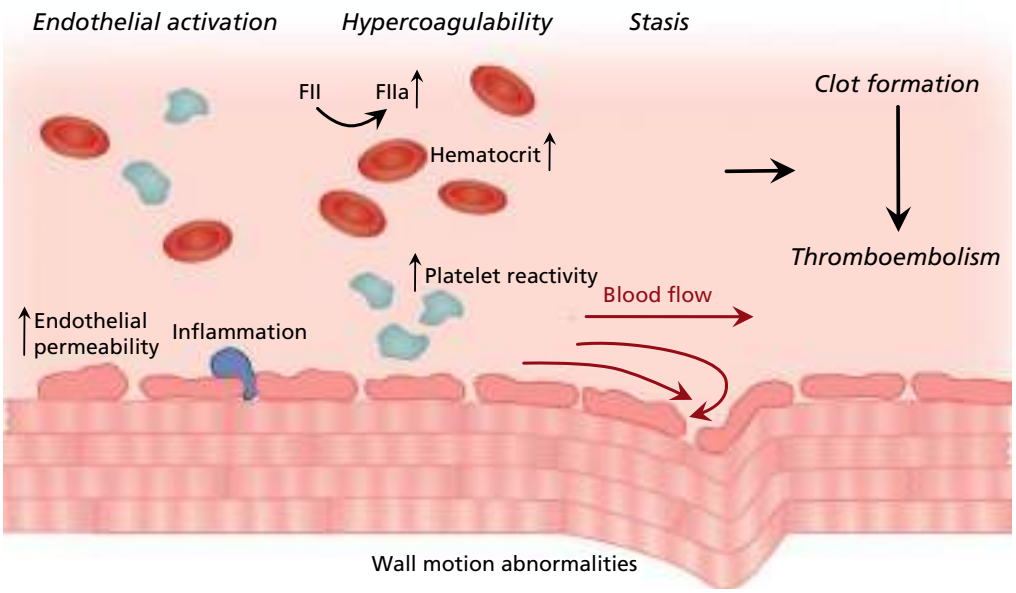

Figure 50.5 captures the main mechanisms involved in thrombogenesis during atrial fibrillation, where the left atrial appendix (indicated at the bottom) is the primary site of thrombus formation. The interaction of the typical Virchov factors (flow, activated endothelium and activated coagulation) precipitate thrombus formation and embolisation that comprises a risk for thromboembolic stroke.

Table 50.1 Risk scores to estimate risk of ischaemic stroke and bleeding respectively, in patients with atrial fibrillation (AF). While based on the CHA2DS2-VASc score an indication for the use of oral anticoagulants is made, the purpose of the bleeding risk assessment is to determine the presence of any modifiable risk factors in order to reduce the bleeding safety hazards related to anticoagulation.

	CHA2DS2-VASc score	score
C	Congestive heart failure or LVEF ≤40%	1
H	Hypertension	1
A	Age ≥75 yr	2
D	Diabetes	1
S	prior Stroke, TIA or thromboembolism	2
V	Vascular disease	1
A	Age 65–74 yr	1
Sc	Sex category (female)	1
	Average to high risk, indication for anticoagulation	≥2
	HAS-BLED score	**score**
H	Hypertension	1
A	Abnormal renal and/or liver function (1 point each)	1–2
S	Stroke	1
B	Bleeding	1
L	Labile INR	1
E	Elderly (≥65 yr)	1
D	Drugs or alcohol (1 point each)	1–2
	High bleeding risk	≥3

commonly assessed with the HAS-BLED score (Table 50.1), which in the PCORI-commissioned systematic review comparing 38 studies with bleeding risk assessment provided the best evidence for predicting bleeding risk (moderate strength of evidence) (Table 50.1). In particular, potentially modifiable risk factors for bleeding like anaemia, renal or liver failure, hypertension and interacting medication, require attention. In the era of VKA, labile INR also was a relevant element and in those with current VKA this is a factor to be addressed; practically, this would mean discussing causes of INR instability (intake, illness, drug interactions, food, adherence) and the need for switching to a DOAC or, in some countries towards a longer acting VKA preparation.

When the joint decision is to prescribe an anticoagulant the choice of a DOAC is preferred over VKA, according to current guidelines. Current DOAC's comprise four different preparations in most countries, including the direct thrombin inhibitor dabigatran and the factor Xa inhibitors rivaroxaban, apixaban and edoxaban. The choice of DOAC depends on several considerations, including preference for once or twice daily dosing, capsule or tablet, expected side effects (e.g. dyspepsia which may occur with dabigatran), drug interactions and renal and liver function. All 4 DOAC's were compared against VKA in large, randomised controlled trials that unequivocally demonstrated non-inferiority with regard to efficacy, i.e. prevention of ischaemic stroke. None of the DOACs have been compared to each other so it remains difficult to assess relative efficacy and in particular safety for bleeding risk for one against the other. Network meta-analyses suggest a favourable bleeding risk profile for apixaban, as compared to others. However for reasons of adherence, once-daily DOACs may also be preferred. The risk of bleeding is highest for gastrointestinal bleeding (±2% annual risk), which is obviously influenced by comorbidity, drug interactions and other factors as estimated in the HAS-BLED score. Recent studies suggest that DOAC pharmacokinetics may be a factor to consider in estimating bleeding risk. DOAC peak anti-Xa levels vary considerably between individuals, due to pharmacodynamic and -kinetic factors and peak levels have been associated with bleeding risk. One reason for variation in bleeding risk amongst DOACs may be that such peak variation is less evident for twice daily dosed DOACs like apixaban, as compared to once daily dosed rivaroxaban. Although DOACs should not be routinely monitored with assays that reflect their plasma level, such testing may be helpful in establishing an individual patient profile, in order to guide the selection of the optimal drug in patients that are vulnerable for any reason (very old age, under or overweight, comorbidity etc.). At least annual surveillance of DOAC use is warranted in all patients focusing on complications, drug adherence, interactions and side effects, laboratory assessment of liver and kidney function. The frequency of such monitoring should be dependent on critical factors that influence decisions on dose adjustment such as kidney function (estimated clearance).

The presence of active gastrointestinal or urogenital cancer is a specific reason to be careful with any form of systemic anticoagulant and the use of DOAC for AF prevention should be frequently monitored in such patients (temporary replacement with LMWH may be needed in case of actively bleeding tumours). The same precautions may play a role in patients with malignancies treated with chemotherapy and anticipating temporary reductions in platelet count; for the management in such conditions, the reader is referred to guidelines from relevant haemato-oncological societies.

Although not the preferred anticoagulants anymore, VKA can still be used for patients with AF, in particular, if the shared decision-making suggests a potential advantage of VKA over DOAC. Such considerations can include cost of medication, expected or experienced side effects, concerns over medication adherence (favouring INR-nurse monitored and supervised anticoagulant treatment), severe renal or liver failure. Thanks to the huge worldwide experience with VKA of more than 70 years, there are few unexpected issues related to VKA that may be encountered and INR control is worldwide established, although not always in anticoagulation clinics.

The effects of antithrombotic therapy on the vessel wall and atherogenesis: clinical relevance?

Antiplatelet agents

Given the multiple roles of platelets beyond thrombosis and haemostasis, beneficial effects of antiplatelet therapy beyond the inhibition of the thrombotic response have been reported. Platelets are actively involved in the inflammatory response. Upon platelet activation, platelets release many pro-inflammatory mediators from their granules, thereby enhancing an inflammatory response. Overall, data from *in vitro,* as well as *in vivo* mouse studies and patient studies point to an overall reduction in the amplification of the inflammatory response by platelets when antiplatelet therapy is administered. The reduction seen in the number of platelet–leucocyte aggregates and the levels of sCD40L, hsCRP and IL-6 upon clopidogrel, prasugrel or ticagrelor treatment in combination with aspirin argue for an anti-inflammatory effect of antiplatelet therapy. In case of P2Y12 inhibition, the anti-inflammatory effect appears to be dependent on the level of P2Y12 inhibition, although P2Y12- independent effects cannot be excluded.

Further, the targets of antiplatelet therapy, COX1 and P2Y12 are not solely expressed by platelets, but can be found in other cells present in the vasculature as well. As such, beneficial effects of antiplatelet drugs on other cells than platelets have been postulated. *In vitro*, there have been indeed reports on the (off-target) effects of aspirin and P2Y12 inhibitors on the function of endothelial cells and smooth muscle cells. Whether these effects are clinically relevant is unclear. Also, data from *in vivo* mouse experiments advocate protection of the vasculature and attenuation of atherosclerosis by aspirin or P2Y12 inhibitors, although here contrasting results have been published as well. In patient studies vascular protective effects of antiplatelet therapy have been demonstrated in ACS patients undergoing PCI, where especially administration of prasugrel pre-PCI improved endothelial function. In a cross-over study design, no evidence was found for adenosine-mediated off-target effects of ticagrelor on endothelial function.

Anticoagulants

Many decades of use of VKA have revealed a few long-term side effects, of which increased vascular calcification may be the most relevant one. These vascular effects are attributable to the inhibition of carboxylation of specific vitamin K-dependent proteins like matrix Gla protein, a protein involved in the inhibition of both medial and intimal calcification. There is strong preclinical evidence for this mechanism and also studies in humans demonstrate increased calcification in the media of large arteries. While calcification in absence of VKA is a prognostic marker for CVD and mortality, there is no evidence yet that VKA-related calcification is harmful to the patient. However, as with any off-target effects, it is unintended and therefore ideally avoided.

In contrast, DOACs do not interfere with vitamin K metabolism and biological effects should be different. There is ample evidence from preclinical studies that administration of a DOAC (whether a direct thrombin or factor Xa inhibitor) delays the onset and progression of atherosclerosis in mice and factor Xa inhibition may even cause reversal of atherosclerotic lesions. While several lines of study are ongoing to establish the molecular biological mechanisms underlying these effects, it seems clear that DOAC by inhibiting specific coagulation proteases inhibits PAR-mediated cell signalling. Clinically, limited evidence from observational studies with DOACs shows diminished calcification and less plaque instability features in patients on DOAC versus VKA. It is too early to conclude that DOACs have protective effects on the human vasculature and further studies are clearly warranted.

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PART VI

Consultative haematology, supportive care and miscellaneous disorders

CHAPTER 51

Haematological aspects of conditions that occur primarily in tropical countries

51

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Introduction

The chapter has been divided into two sections; the first section covers parasitic infections in which the organisms can be visualised in the blood or bone marrow and the second section covers secondary haematological abnormalities associated with infections. Knowing about the haematological aspects of tropical diseases is important because haematologists are increasingly involved in managing patients who have travelled to, or are from, tropical regions.

In 2019, 3.5% of the world's population lived outside their country of origin. The majority were economic migrants, often moving from tropical countries to temperate ones. Data from the UK Office for National Statistics indicate that in the UK long-term net migration (i.e. immigration and emigration) has remained stable since the end of 2016. In the year ending June 2019, 609,000 people moved to the UK with an intention to stay for 12 months (i.e. prior to COVID-related travel restrictions). Worldwide travel means that haematologists need not only to know about the tropical diseases that can cause haematological abnormalities, but also be able to take relevant travel histories from patients and appropriately interpret haematological results obtained from investigations. They also need to be aware of ethnic variations in reference ranges to avoid wasting resources on unnecessary investigations and to avoid causing undue anxiety for the patient.

Ethnic variations in reference ranges

The white blood cell count and relative and absolute neutrophil counts are lower in people of African and Middle Eastern descent than in Caucasians. After the age of 1 year, Africans have lower counts than West Indians or African Americans (Table 51.1). The difference is due to a greater number of neutrophils in the storage pool of the non-Caucasian populations. Stimulation of a neutrophil response in these ethnic groups leads to rises in the neutrophil count to the same level as white populations, irrespective of the baseline level. Indian, Chinese and Southeast Asian populations have the same white blood cell and neutrophil counts as northern Europeans. The platelet counts in healthy West Indians and Africans may be 10–20% lower than in Europeans living in the same environment. It is not clear whether there are true ethnic variations in eosinophil counts, but counts of up to 2×10^9/L have been described in healthy blood donors in Africa. Interpretation of variations in blood counts should therefore take account of the individual's ethnic background. It must be borne in mind that 'normal' values derived from populations

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Table 51.1 Automated white cell (WBC) and neutrophil counts in adults of different ethnic origins (95% ranges).

	Male		Female	
	WBC ($\times 10^9$/L)	Neutrophil count ($\times 10^9$/L)	WBC ($\times 10^9$/L)	Neutrophil count ($\times 10^9$/L)
Caucasian	3.7–9.5	1.7–6.1	3.9–11.1	1.7–7.5
Afro-Caribbean	3.1–9.4	1.2–5.6	3.2–10.6	1.3–7.1
African	2.8–7.2	0.9–4.2	3.0–7.4	1.3–3.7

with high background prevalence of ill health may include individuals with asymptomatic conditions.

Parasitic diseases with organisms in peripheral blood or bone marrow

Malaria

Epidemiology and biology

Until 2004, only four species of plasmodia were known to cause malaria in humans: *Plasmodium (P) falciparum*, *P. vivax*, *P. ovale* and *P. malariae*; since then there have been increasing numbers of cases of infection by a fifth plasmodium, i.e. *P. knowlesi* predominantly reported from Malaysia, Malaysian Borneo and Thailand. *P. falciparum* remains by far the most dangerous and causes the greatest mortality and morbidity (see Figure 51.1).

The total number of cases of malaria imported into the UK has remained stable at around 1500/year since 2000. Approximately 80% of *P. falciparum* infections in the UK are acquired in sub-Saharan Africa and 80% of *P. vivax* infections are acquired in South Asia, especially India and Pakistan. In 2018, 1683 cases of malaria were reported in the UK, of which 1375 (82%) were due to *P. falciparum,* 143 (8.5%) due to *P. vivax* and 117 (7%) due to *P. ovale*. The rest were due to *P. malariae (36) P. knowlesi* (2) or mixed/unspecified (10). There were six deaths. Around a third to a half of all malaria cases in the UK are due to a visit to families in countries of origin and many of these travellers have not taken malaria prevention tablets. Malaria has also been reported in travellers who have transited through airports in malarious areas.

Malaria is transmitted by the bite of an infected female *Anopheles* mosquito. The infecting agent is the spindle-shaped sporozoite and thousands of these may be injected by a single bite. Infrequently, transmission may also occur through blood transfusion, bone marrow transplantation and transplacentally (0.5% of all UK cases). There have also been reports of malaria transmission in aircraft or near airports in temperate zones due to infected mosquitoes being brought to non-malarious areas.

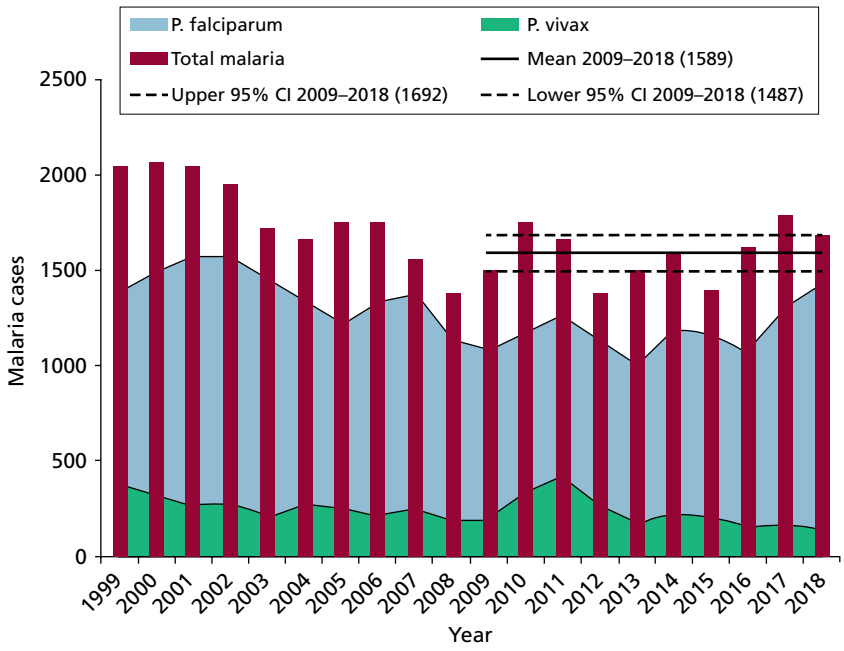


Figure 51.1 Cases of malaria in the United Kingdom: 1999–2018 (Source: Data from the PHE Malaria Reference Laboratory.)

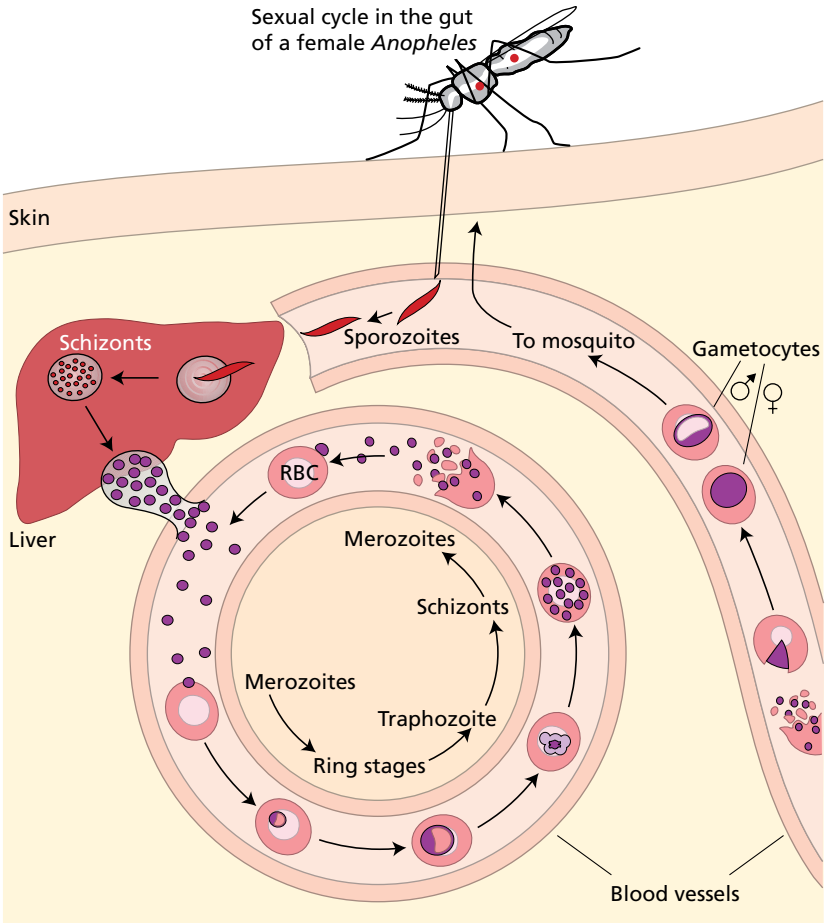


Figure 51.2 Life cycle of the malaria parasite.

P. knowlesi is transmitted by *Anopheles latens*. It does not occur in Africa because its reservoir hosts, long-tailed and pig-tailed macaques, are not found in Africa.

Within a few hours of an infected bite, the sporozoites enter the hepatocytes, where they divide (Figure 51.2). Rupture of the hepatocyte releases the parasites into the blood, where they attach to red cell membranes using specific receptors. In the red cells, asexual replication of the trophozoites (ring forms) occurs, giving rise to erythrocyte schizonts. The schizonts mature into merozoites and are released into the circulation to re-infect other red cells. The periodicity of this release varies with the species and is responsible for the classical cyclical nature of malaria fevers. Relapses, which can occur months or years after the primary illness, are characteristic of infection with *P. vivax* and *P. ovale*, and are due to maturation of persistent hypnozoites in the liver.

A few of the trophozoites develop into male and female banana-shaped gametocytes and are taken up by the mosquito at a blood meal. Inside the midgut of the mosquito, they undergo sexual reproduction and sporozoites migrate to the salivary glands, ready to infect another host when the mosquito bites. Unlike the schizonts of *P. vivax*, *P. ovale P. malariae* and *P. knowlesi*, those of *P. falciparum* are not commonly seen in the peripheral blood of the human host. This is because *P. falciparum*-infected cells have surface cytoadherence molecules that enable them to be sequestered in the deep tissues. Therefore *P. falciparum* schizonts only appear in the blood in very severe infections or in splenectomised patients. Sequestration is responsible for some of the severe clinical consequences of *P. falciparum* malaria, such as cerebral malaria.

Clinical features

The time between the infected bite and the appearance of clinical symptoms and parasites in the peripheral blood varies between species. It is 7–30 days (mean 10 days) in *P. falciparum,* but can be months, or even years, with other species, particularly *P. vivax* and *P. ovale*, because of their hypnozoite stage.

All five *Plasmodium* species produce factors that cause release of tissue cytokines, which produce fever and contribute to anaemia through marrow suppression. Splenomegaly is a common feature of acute malaria and mild jaundice may also occur secondary to haemolysis. Other clinical features vary with different

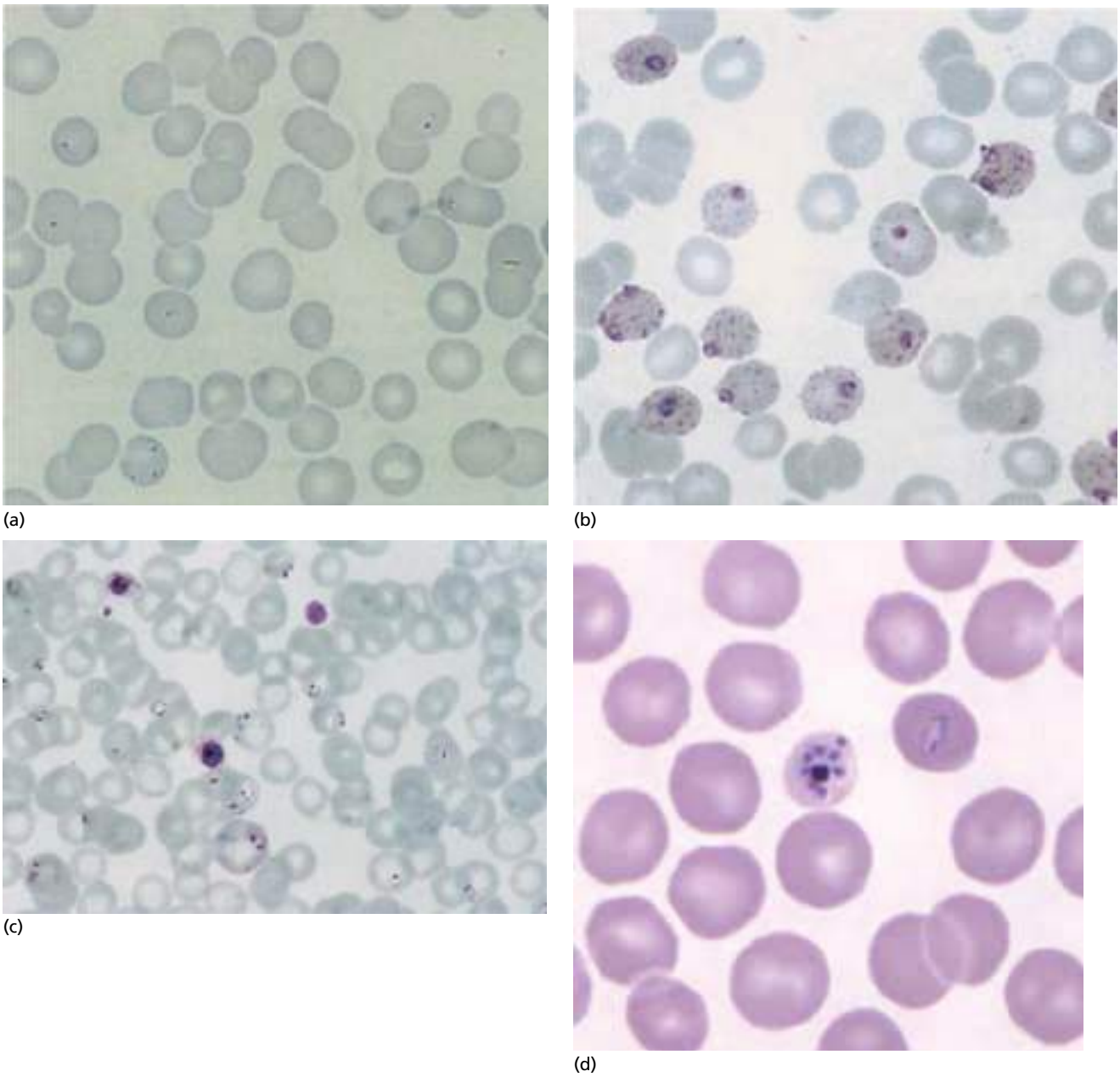


Figure 51.3 Stages in the life cycle of *Plasmodium falciparum* in Giemsa-stained thin films; the cells are not enlarged or decolourised: (a) delicate early ring forms, (b) ring forms with prominent Maurer's clefts and (c) ring forms and early and late schizonts (schizonts are not commonly seen in the peripheral blood). (d) Ring form and schizont of *Plasmodium knowlesi* (Part (d) Source: GEFOR (2012). Reproduced with permission of Galería de imágenes y recursos.)

species. Maximal immunity to malaria takes around 10 years to develop and is lost over the course of 1–5 years if the individual leaves a malarious area and is no longer exposed to infections.

Plasmodium falciparum

Plasmodium falciparum is the most common species associated with complicated and severe disease (Figure 51.3) though *Plasmodium vivax* has also been associated with severe and fatal disease. In *P. falciparum* infections, death may occur after a single exposure to malaria, particularly in those with no immunity, such as non-immune travellers or young children in endemic countries. Recurrent attacks with different strains lead to the development of clinical immunity, but not necessarily to complete clearance of parasites. Parasites

Table 51.2 Clinical features of *P. falciparum* malaria infection.

Target organ	Clinical features	Potential common misdiagnosis
Gastrointestinal	Diarrhoea, vomiting	Traveller's diarrhoea
Respiratory	Cough, pulmonary oedema	Pneumonia, cardiac failure
Neurological	Delirium, coma, convulsions, focal neurological signs	Encephalitis, meningoencephalitis
Renal	Oliguria, haemoglobinuria	Nephritis
Hepatic	Jaundice, hypoglycaemia	Hepatitis
Haematological	Anaemia, splenomegaly	Viral infection, lymphoma

may therefore be detected in a high proportion of clinically asymptomatic adults in endemic areas. In addition to fever with rigours, nausea and hot and cold phases, *P. falciparum* infection can also present with diarrhoea and cough (Table 51.2). Serious complications include severe anaemia, cerebral involvement, and kidney and liver failure.

During pregnancy, immunity to malaria is reduced and parasite density increases. Even when parasites cannot be visualised in the peripheral blood, they may be sequestered in the placenta and compromise fetal development. Malaria is an important cause of low birth weight in neonates and anaemia in pregnant women, both of which have a detrimental effect on the later development of the infant.

Plasmodium malariae

The incubation period of *P. malariae* may be several weeks. It is associated with recurrent fever, anaemia and enlargement of the liver and spleen. Without treatment or following incomplete treatment of the primary infection, re-crudescences may occur with decreasing severity over many years. Clinical symptoms of malaria have been reported up to 30 years after the initial infection.

Plasmodium vivax and *Plasmodium ovale*

These species cause a similar clinical picture with bouts of fever occurring periodically up to 5 years after the initial infection. These are relapses due to reinvasion of red cells by hypnozoites. The trigger for hypnozoites to reactivate after dormancy is unknown.

Plasmodium knowlesi

The incubation period of *P. knowlesi* infection is about 11 days. It causes severe, and occasionally fatal, malaria. This is because the parasite invades red cells of all ages and has a short life cycle of 24 hours.

Haematological abnormalities

Normochromic normocytic anaemia is common in malaria, particularly in children, but the degree and rapidity of onset are variable. The haemoglobin may fall by up to 20 g/L each day. In malaria-endemic regions, chronic anaemia due to nutritional deficiencies, intestinal helminths, HIV infection and haemoglobinopathies may be compounded by anaemia due to malaria. In chronically anaemic patients, the oxygen dissociation curve is shifted to the right so they are better able to tolerate further falls in haemoglobin. The clinical effects of anaemia in malaria are therefore due to a combination of the degree and rate of fall of haemoglobin.

Anaemia due to malaria has multiple aetiologies: red cells containing parasites are removed from the circulation by the reticuloendothelial system; there is accelerated destruction of non-parasitised cells and dyserythropoiesis in the bone marrow. Both parasitised and non-parasitised red cells lose deformability and the high shear rates in the spleen enhance their removal. In acute malaria, reticulocyte response is suppressed and erythropoietin levels are elevated, although this may be less than expected for the degree of anaemia.

Uncommon complications of malaria that can exacerbate the anaemia are hyper-reactive malarial splenomegaly (HMS) and 'blackwater fever'. HMS is characterised by massive splenomegaly with hypersplenism (see later) and occurs as a result of a disordered immune response to malaria. Blackwater fever is characterised by severe intravascular haemolysis with haemoglobinuria and can lead to acute renal failure. It has most commonly been associated with malaria, antimalarial drugs (particularly quinine) and glucose-6-phosphate dehydrogenase (G6PD) deficiency.

The high prevalence of severe anaemia in areas with intense malaria transmission has generally been ascribed to malaria. However, studies in Malawian children have shown that the anaemia is multifactorial. In these children, bacteraemia, malaria, hookworm, HIV and deficiencies of G6PD, vitamin A and vitamin B12, but not iron and folate, deficiency, were associated with anaemia.

Case fatality rates of children with severe anaemia in Africa are 9–18% and mortality rise steeply at haemoglobin concentrations of less than 40 g/L. Severe decompensated malarial anaemia can be accompanied by hypovolaemia and acidosis and therefore requires careful rehydration (avoiding rapid fluid boluses which may be harmful), and blood transfusion. Since there are risks associated with transfusions, it is important to prevent and adequately treat mild and moderate anaemia to

minimise transfusions. Antimalarial prophylaxis, prompt treatment of malaria and avoidance of mosquito bites (for example by using insecticide-impregnated bed nets and topical repellants) are valuable in reducing malaria infections and anaemia, especially in vulnerable groups, such as pregnant women and young children.

The white cell count in malaria is usually normal but may be raised in severe disease. Other white cell changes that have been described in malaria include a leucoerythroblastic response, monocytosis, eosinopenia and a reactive eosinophilia during the recovery phase. Neutrophil activation may be apparent in severe malaria. Thrombocytopenia due to increased splenic clearance, and thus increased platelet turnover and raised thrombopoietin levels is usually mild with counts around 100×10^9/L, but can be marked in *P. knowlesi* infection. Pancytopenia without massive splenomegaly has also been described.

The bone marrow of patients with acute malaria due to any of the species shows prominent dyserythropoiesis. This may persist for weeks after the acute infection and is caused by intramedullary cytokines produced by the infection. Erythrophagocytosis and macrophages containing malaria pigment are frequently seen in marrow samples.

Although malaria is associated with thrombocytopenia and activation of the coagulation cascade and fibrinolytic system, bleeding and haemorrhage are uncommon even though the prothrombin and partial thromboplastin times may be prolonged. Clinically apparent bleeding or disseminated intravascular coagulation is associated with very severe disease and a high mortality. Severe *P. falciparum* infection is associated with endothelial cell activation, abnormal circulating ultra large vWF multimers and reduced plasma ADAMTS13 function. These changes may result in intravascular platelet aggregation, thrombocytopenia and microvascular disease. Fibrinogen levels are often increased and there is rapid fibrinogen turnover with consumption of antithrombin and factor XIII resulting in increased fibrin degradation products. Microparticle formation from platelets, red cells and macrophages also causes widespread activation of blood coagulation. Haematological indicators of a poor prognosis in severe malaria include:

- leucocytosis $>12 \times 10^9$/L;
- severe anaemia (packed cell volume <15%);
- thrombocytopenia $<50 \times 10^9$/L;
- prolonged prothrombin time (>16 s);
- prolonged partial thromboplastin time (>40 s);
- reduced fibrinogen (<2 g/L);
- hyperparasitaemia >100,000/μL (high mortality >500,000/μL);
- >20% of parasites are pigment-containing trophozoites and schizonts;
- >5% of neutrophils contain visible malaria pigment.

Effective anti-malarial agents are important in the treatment of coagulopathy in malaria. Antiplatelet agents should be avoided since they interfere with the protective effect of platelets against malaria.

Genetic haematological mechanisms that protect against severe malaria

Plasmodium vivax and *P. knowlesi* need Duffy blood group antigen as a receptor to enter red cells. This antigen is absent in at least two-thirds of all Africans who consequently have a natural resistance to infection with *P. vivax and P. knowlesi*. Hb AS has a substantial (up to 10 times) protective effect against severe malaria and a similar, but less marked protection is associated with other genetic red cell abnormalities such as G6PD deficiency, thalassaemia trait and Hb C trait.

Diagnosis

Microscopy and rapid diagnostic tests (RDTs) are the primary choices for diagnosing malaria in the field, but they cannot detect low-density malaria infections.

Microscopy

Direct visualisation of parasites by light microscopy using a combination of thick and thin blood films is the gold-standard diagnostic technique for malaria (Table 51.3 and Figure 51.3). A Romanowsky stain (e.g. Field, Giemsa and Leishman) pH 7.2 is used so that the parasite cytoplasm stains blue and the nuclear chromatin stains red. A thick blood film examined by two observers, each viewing a minimum of 200 high-power fields, should be used as the first screening tool as it allows larger volumes of blood to be examined than the thin film. However, the parasites appear distorted in thick films due to the process of lysing the red cells so this method cannot be used for parasite morphology and speciation. A thin blood film should be performed on any sample that yields a positive or uncertain thick film result. This allows visualisation of undistorted parasites and an assessment of the size and shape of the red cells, so speciation and quantification (parasites/μL) can be carried out. However, the thin film has low sensitivity because of the small amount of blood that can be examined.

The disadvantages of basing a diagnosis of malaria on blood film examination include:

- An initial negative film does not exclude malaria: at least three films taken during episodes of fever should be examined in the absence of antimalarial drugs to confirm a negative blood film.
- A positive film does not prove that symptoms are due to malaria: asymptomatic parasitaemia is common in adults from endemic areas.
- *P. malariae* and *P. knowlesi* cannot be distinguished morphologically.
- Parasites, particularly *P. falciparum* gametocytes, may be washed off the slide during staining and bulk staining may result in transfer of parasites between slides.
- Parasite density does not necessarily correlate with disease severity, although heavy parasitaemia (>5% of red cells infected) indicates a poor prognosis.

Table 51.3 Differentiating epidemiological and stained thin blood film features of the five *Plasmodium* species affecting humans compared to *Babesia* species.

	P. falciparum	*P. knowlesi*	*P. vivax*	*P. ovale*	*P. malariae*	*Babesia* sp.
Main site of distribution, proportion in the endemic area (%)	Global, most common in Africa, 80–90 (Africa)	South-east Asia,1–60	Mostly in Asia, 50–80	Africa, 5–8	Global, 0.5–3	
Life-threatening	Yes	Yes	Yes	No	No	
Parasitemia	Can be high	Can be high	<2%	<2%	<2%	
Treatment	Combinations with arthemisinine derivatives: atovaquone plus proguanil; mefloquine; quinine +/– doxycycline	Probably all the medications listed for the other plasmodia	Chloroquine followed by primaquine	Chloroquine followed by primaquine	Chloroquine	
Appearance of infected red blood cells (size and shape)	Normal size and shape	Normal size and shape although some have irregular edges	1.5–2 times larger than normal; shape normal or oval	As for *P. vivax*, but some have irregular edges	Normal shape; size normal or smaller	Both normal
Red cells with multiple parasites/cell	Common	Common	Occasional	As for *P. vivax*	Rare	Common
Stages present in peripheral blood	Rings and gametocytes; occasionally schizonts	All stages: late stages similar to *P. malariae*	All stages	As for *P. vivax*	All stages	Only rings and rare pear-shaped forms ('Maltese cross'); no gametocytes
Ring form (young trophozoite)	Delicate small ring; scant cytoplasm; sometimes at the edge of red cell ('accolé form')	Similar to *P. falciparum*	Ring one-third to half of the diameter of cell; heavy chromatin dot	As for *P. vivax*	Ring one-third of the diameter of cell; heavy chromatin dot; vacuole sometimes 'filled in'	Resembles ring of *P. falciparum*; look for pear-shaped structure
Gametocyte	'Crescent' or 'sausage' shape is characteristic	Similar to *P. malariae*; comprise 1–3% of infected erythrocytes	Round or oval	Round or oval (smaller than *P. vivax*)	Round or oval; dark coarse pigment	No gametocyte

Malaria pigment may persist in phagocytic cells for several weeks after an acute attack and may be helpful in retrospective diagnosis of malaria. Automated haematology analysers may produce an abnormal pattern on the white cell differential count histogram. Debris below the white cell threshold may be due to malaria parasites and manual examination of blood films is indicated if this pattern is flagged up by the analyser.

Malaria rapid diagnostic tests (antigen detection)
Malaria RDTs detect specific antigens produced by malaria parasites (e.g. histidine-rich protein 2 or parasite lactate dehydrogenase) in the blood of infected individuals. Since 2018 evaluations of RDTs have been managed by the WHO. There are >200 RDTs available: some detect a single Plasmodium species, some detect multiple species and others can distinguish between *P. falciparum* and non-*P. falciparum* infection, or other species. Blood for the test is obtained from a finger-prick and results are available within 15–30 minutes. The drawbacks of RDTs are false positives because HRP2 persists in the blood for several days after infection clearance and false negatives due to gene deletions. RDTs have a sensitivity of ~100 parasites/μL though new 'ultra-sensitive' RDTs may be helpful in distinguishing between active versus recently cleared malaria infections.

Antibody detection
Malarial antibodies can remain in the blood after the eradication of parasites, so their detection is not useful for diagnosis in the acute attack. The main uses of malarial antibody detection are for excluding malaria as a cause of recurrent fever, for population surveys and as a screening test for blood donors in non-endemic areas.

Nucleic acid amplification tests
Nucleic acid amplification tests (NAATs) can detect low-density malaria infections (<1 parasite/μL). They can quantify parasite load and detect sexual- and asexual-stage parasites. Currently, WHO recommends that NAATs should only be used for research until more evidence is available on the public health impact of detecting and treating low-density infections.

Haematological implications of treatment for malaria

Currently, the recommended first-line treatment for uncomplicated falciparum malaria, for mixed infections and for chloroquine-resistant vivax malaria is artemisinin-based combination therapy. The following drugs used for treating malaria may have haematological side effects.

Pyrimethamine is used in combination with a long-acting sulfonamide, such as sulfadoxine (as in Fansidar), a dihydrofolate reductase inhibitor, which may therefore induce megaloblastic anaemia and other cytopenias. The sulfur component of these combinations may also cause methaemoglobinaemia.

Dapsone acts by inhibiting the synthesis of dihydrofolic acid and is used as part of a fixed combination with proguanil or chlorproguanil. It may be associated with haemolytic anaemia, methaemoglobinaemia and eosinophilia.

Primaquine, an inhibitor of protein synthesis, is active against the hypnozoites of *P. vivax* and the gametocytes of *P. falciparum*. It causes oxidant haemolysis in patients with G6PD deficiency and, rarely, methaemoglobinaemia.

Quinine is usually reserved for life-threatening infections, especially in endemic countries. It acts by disrupting the food vacuole of the parasite. Rarely, it is associated with immune thrombocytopenia and severe intravascular haemolysis.

Mefloquine, halofantrine and artemisinin-related compounds do not commonly cause significant haematological side effects. A combination of artemether and lumefantrine (Coartem or Riamet) is commonly used as first-line treatment in endemic areas.

Some antibacterial drugs, such as doxycycline, clindamycin, trimethoprim and sulfonamides, have also been used for their antimalarial effect and may be associated with haematological side effects.

For malaria due to *P. vivax*, *P. ovale, P. malariae and P. knowlesi*, chloroquine is still widely used for treatment, as resistance is generally low; primaquine is added to prevent relapses in vivax, ovale and knowlesi malaria.

Anti-coagulation and malaria chemoprophylaxis

Travellers on warfarin should begin malaria chemoprophylaxis one week (2–3 weeks for mefloquine) before travelling and the INR should be stabilised before travel. There is limited experience of chemoprophylaxis in people taking direct-acting oral anticoagulants.

Babesiosis in the differential diagnosis of malaria

Babesiosis can be confused clinically and haematologically with malaria, presenting with fever and haemolysis. It occurs worldwide as an infection of livestock occasionally infecting humans, and clinical babesiosis has been reported in both immunocompromised and immunocompetent patients. There are more than 100 species. It is endemic and increasing in the north-eastern and upper midwestern regions of the USA particularly affecting middle-aged and elderly white males. It is found sporadically in other parts of the USA, Europe, Asia, Africa and South America. It is due to a protozoan parasite transmitted by the bite of the ixodid tick. Following the bite,

the organisms penetrate red cells, where they take on an oval, round or pear shape and multiply by budding. The erythrocytic ring forms of *Babesia microti* and *B. divergens* may be confused with malaria *P. falciparum* rings, but they do not produce pigment or cause alterations in red cell morphology. A minority of organisms take on a folded shape and are thought to be gametocytes.

Babesia bovis, *B. microti* and *B. divergens* are responsible for the majority of human infections. The consequences of babesiosis can range from asymptomatic infection to severe and occasionally fatal outcomes. Most of the cases reported from Europe have been due to *B. divergens* and occurred in patients without a functioning spleen. In North America, almost all the cases have been due to *B. microti*, which usually produces a subclinical infection but can be life-threatening and occasionally fatal. Transfusion-transmitted cases have been reported in the US. Specific laboratory diagnosis of babesial infection is made by morphological examination of Giemsa-stained blood smears, serology and amplification of babesial DNA using polymerase chain reaction.

Filariasis

Epidemiology and biology

There are two groups of human filariasis: those that occur in the blood (lymphatic filariasis) and those that occur in the skin (onchocerciasis). Only lymphatic filariasis is considered in this chapter, as it is associated with detectable organisms in the peripheral blood.

Three species of filarial worms cause lymphatic filariasis in humans, *Wuchereria bancrofti, Brugia timori* and *Brugia malayi*. They have different geographical distributions, with *W. bancrofti* being the most widespread. More than 90% of infections due to *W. bancrofti* are found in Asia. It also occurs in Africa, America and the Pacific islands. Filariasis due to *B. malayi* occurs in China, Indo-China, Thailand, Malaysia, Indonesia, the Philippines and southwest India, *Brugia timori* has predominantly been reported in Indonesia (Figure 51.4).

The worms are 4 mm (male) to 10 mm (female) in length and can live for over 10 years in the lymphatics. Microfilariae, which are 250–300 µm long, are produced by the female worm

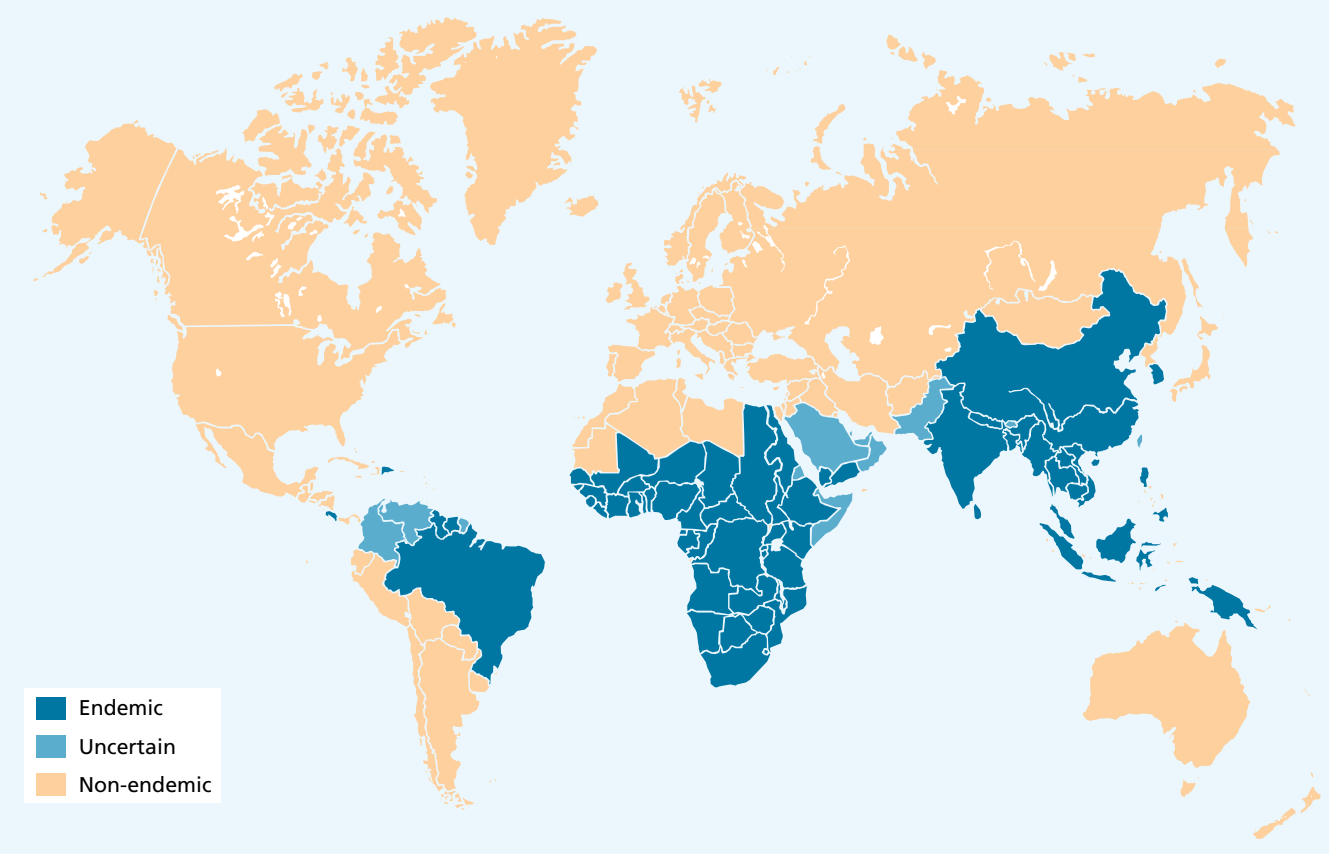


Figure 51.4 Global distribution of lymphatic filariasis.

and released into the blood after 3–8 months, where they may live for up to 1 year. Microfilariae densities can reach 10,000/mL but are usually much lower. They exhibit daily periodicity in the blood and this timing is designed to match the biting habits of their mosquito vectors, culicine and anopheline mosquitoes. The microfilariae develop into infective larvae in the mosquito and pass into the proboscis, ready to be transmitted into a new host.

Clinical presentation

There is wide variation in the presenting features of lymphatic filariasis, which may occur 6 months or more after the infective bite. The symptoms and signs are due to lymphangitis. There are recurrent bouts of fever with heat, redness and pain over lymphatic vessels. In fair-skinned people, the lymphangitis can be seen to spread distally (i.e. the opposite direction to septic lymphangitis). In *W. bancrofti* infection, these repeated episodes of inflammation eventually result in the typical chronic picture of filariasis, including hydrocele, lymphoedema and elephantiasis, chyluria and tropical pulmonary eosinophilia. The clinical picture in *B. malayi* infection is similar, but it does not cause hydrocele or chylous urine. *B. timori* causes lymphoedema characteristically limited to the leg below the knee.

Other filariae with blood-inhabiting larvae

Loa loa

This occurs in the rainforest belt of Africa, especially West Africa. The adult worms migrate through the subcutaneous tissues, including the conjunctiva, and occasionally can be seen passing across the eye.

Mansonella perstans

This is a common infection in Africa. It can cause angioedema, pruritus, abdominal pain and eosinophilia. These organisms often coexist in the blood with *W. bancrofti*, but can be distinguished by their smaller size and absence of a sheath.

Mansonella ozzardi

This is probably non-pathogenic and occurs in the West Indies and South America. The adult worm lives in the body cavities and mesentery, rarely causing any symptoms.

Haematological abnormalities in filariasis

Eosinophilia is the major and most frequent haematological abnormality found in lymphatic filariasis. Tropical pulmonary eosinophilia is a complication of filariasis and is due to an immunological hyper-responsiveness to microfilariae in the lungs. It is more common in men than women. Although microfilariae are absent from the blood in this syndrome, they may be seen in lung biopsies and adult worms can be visualised in lymphatics on ultrasound. There is an extreme eosinophilia, with eosinophil counts of greater than 10×10^9/L; the level of eosinophilia is not related to the severity of symptoms. A study of filariasis in India in 2013 found that, in addition to eosinophilia, there was leucocytosis, neutrophilia, thrombocytosis, raised erythrocyte sedimentation rate and raised mean corpuscular volume. Haemoglobin, total red cell count, lymphocytes, basophils, monocytes, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration was reduced. In tropical pulmonary eosinophilia, diethylcarbamazine treatment reduces the eosinophil count and produces resolution of symptoms. This rapid response to treatment distinguishes filariasis from other causes of marked eosinophilia associated with helminths such as *Ascaris*, *Strongyloides*, *Schistosoma* subsp. *trichinosis* and *Toxocara*.

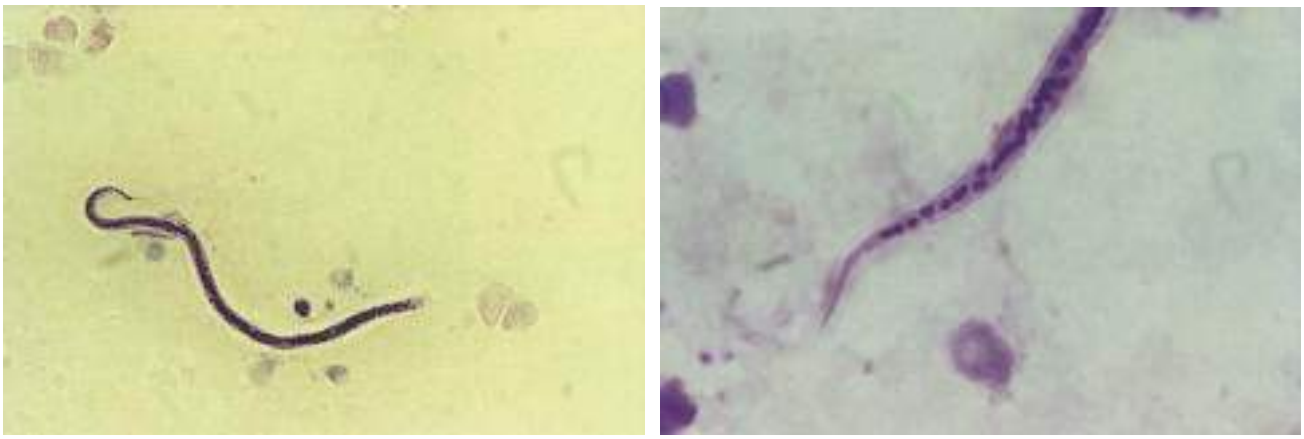
Diagnosis of filariasis

Diagnosis is based on finding microfilariae in the peripheral blood. The level of filaraemia is inversely related to the clinical signs because much of the damage is due to immunological responses to the microfilariae rather than to the organisms themselves. Furthermore, the presence of microfilariae does not necessarily mean that they are causing clinical problems and, conversely, a lack of microfilariae in the blood does not exclude a diagnosis of filariasis. The peripheral blood findings must therefore be assessed in the context of the clinical picture.

To optimise the chances of finding scant microfilariae in the blood, the sample should be taken at the appropriate time for the expected peak concentration of microfilariae (i.e. around midnight or midday for nocturnally and diurnally periodic forms, respectively). There are many techniques for demonstrating microfilariae in the laboratory. The simplest method is a wet preparation of fresh blood. Microfilariae will survive in venous blood collected into EDTA for 2 days at room temperature. Motile microfilariae can be seen on a slide under low power and can be counted in a counting chamber. Numbers of microfilariae may be low, requiring concentration techniques such as blood filtering.

For species identification, thick and thin blood films should be stained with Giemsa or haematoxylin and the microfilariae differentiated according to the pattern of their sheaths, nuclei distribution and size (Figure 51.5). The edges of the film should be examined carefully as microfilariae tend to be concentrated at the periphery and are easily missed unless the whole film is scanned at low magnification.

Detection of circulating antigen by enzyme-linked immunosorbent assay (ELISA) or immunochromatography (ICT) has replaced microscopy for the diagnosis of bancroftian, but not brugian, filariasis. An antigen ICT card test is available for the



(a) (b)

Figure 51.5 Microfilariae of *W. bancrofti* in thick film: (a) microfiliaria showing the negative impression of the sheath (× 365); (b) tail of the microfilaria showing that the nuclei do not extend into the tail (× 912).

detection of *W. bancrofti*, which does not react with other filariae and is highly sensitive (100%) and specific (92%). Filarial DNA can be detected by PCR, and ultrasound scans can help identify adult worms within the lymphatic system. Serological tests are not very helpful as most individuals from endemic areas have antibodies to crude filarial antigens and there is cross-reactivity with other filariae and nematodes.

Haematological implications of treatment for filariasis

Oral diethylcarbamazine is the drug of choice in all forms of lymphatic filariasis, including subclinical infection. Alternative treatments include combinations of albendazole and ivermectin. None of these drugs has common, serious haematological side effects. Depletion of *Wolbachia* endobacteria, a symbiont of *Onchocerca*, by tetracycline antibiotics leads to long-lasting sterility of adult female worms.

African trypanosomiasis (sleeping sickness)

Epidemiology and biology

African sleeping sickness is caused by the haemoflagellate protozoa *Trypanosoma brucei gambiense* in West and Central Africa, and *T. brucei rhodesiense* in eastern Africa (Figure 51.6). These parasites are fusiform in shape, 12–35 µm long and morphologically indistinguishable from each other. The disease is transmitted by the bite of the tsetse fly, which is only found in Africa. The trypanosomes multiply by fission in the vicinity of the infected bite and are then disseminated by the bloodstream. Congenital transmission has also been described.

The distribution of African sleeping sickness is determined by the ecological limits of the tsetse fly vector and lies in the region between Senegal and Somalia (Latitude 14° north) and the Kalahari and Namibian deserts (Latitude 20° south). Sleeping sickness is an important public health problem in Central African Republic, Chad, Democratic Republic of Congo, Côte d'Ivoire, Guinea, Malawi, Uganda and United Republic of Tanzania. During recent epidemic periods, the prevalence reached 50% in several villages in the Democratic Republic of Congo, Angola and southern Sudan. Improved surveillance has consistently reduced the number of new cases over the last two decades. For the first time in 50 years, the number of reported cases dropped below 10,000 (9878) in 2009 and the decline has continued; 3796 cases were recorded in 2015, the lowest since data collection started 76 years ago.

Clinical features

The bite of a tsetse fly is very painful and causes a small indurated lesion that may persist for some days. The local multiplication of the trypanosomes may cause a marked inflammatory reaction (a chancre) that regresses after 2–3 weeks. Entry of the trypanosomes into the bloodstream is associated with fever, which tends to be less marked in West African trypanosomiasis than in the East African variety. East African trypanosomiasis is primarily a disease of cattle and only enters human hosts by accident. It has a more aggressive course and severe symptoms than West African sleeping sickness.

The early stages of sleeping sickness can be associated with prominent lymphadenopathy, particularly of the posterior cervical nodes, and mild splenomegaly. These features may be suggestive of infectious mononucleosis, tuberculous lymphadenitis or a lymphoproliferative disorder. Severe anaemia, haemorrhages and petechiae may occur at this stage.

Both types of African sleeping sickness cause a protracted febrile illness which, despite the name, is not always associated with drowsiness. Death is inevitable if the disease is left

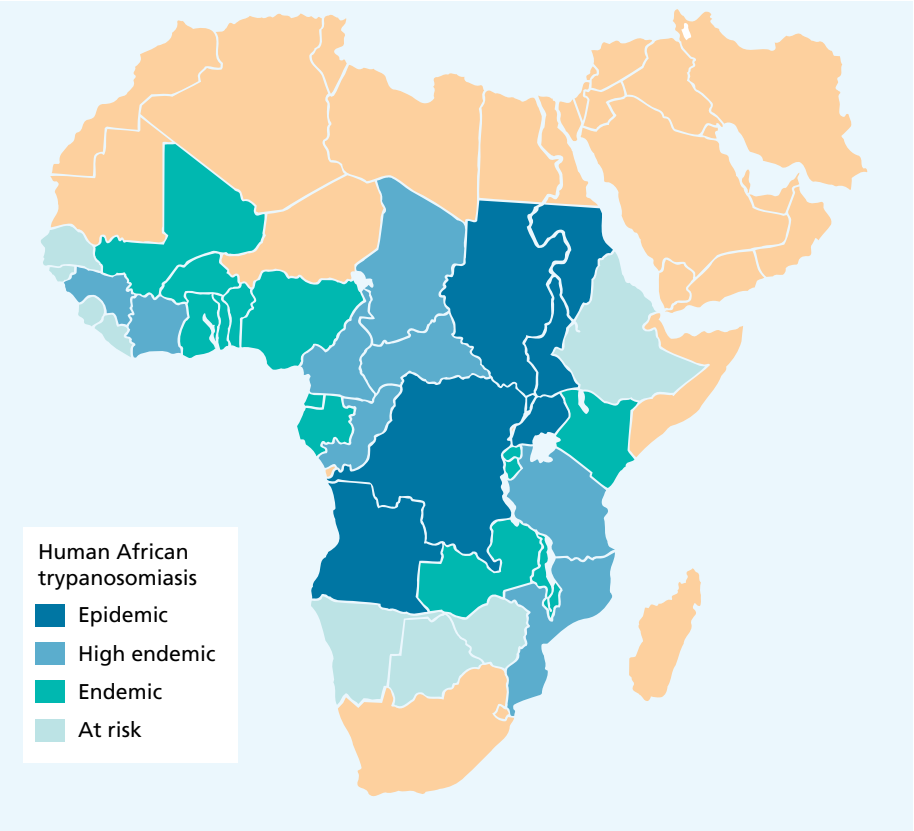


Figure 51.6 Global distribution of African trypanosomiasis.

untreated. As the disease progresses, parasitaemia decreases, trypanosomes invade the central nervous system (CNS) and neurological disturbances due to inflammatory chronic meningoencephalitis supervene. In West African trypanosomiasis, the disease runs its course over several years, but in East African trypanosomiasis infection CNS involvement may occur within weeks.

Haematological abnormalities

The aetiology of the anaemia in sleeping sickness is multifactorial, but primarily due to phagocytic removal of immune complex-coated red cells from the circulation. Trypanosomes liberate haemolytic factors that contribute to this process, and increases in plasma volume cause a dilutional anaemia. There is a failure to incorporate iron into red cell precursors and the resulting dyserythropoiesis means that the bone marrow is unable to compensate for the fall in haemoglobin. There may be a moderate leucocytosis with increased monocytes, lymphocytes and plasma cells. Mott morular cells have also been described as sleeping sickness. The bone marrow is hypercellular, focal loss of haematopoietic cells and deposition of extracellular gelatinous substances, which histochemically are mucopolysaccharides, rich in hyaluronic acid.

As the disease advances, a bleeding tendency may develop due to thrombocytopenia, vascular injury and coagulopathy. Platelet dysfunction has also been described and is manifest as clumping and abnormal aggregation responses. DIC with raised FDPs may occur in the later stages. Although some of these haematological changes can be linked to the non-specific polyclonal activation of B cells, overall the underlying mechanisms are not well understood.

Diagnosis

Trypanosomes can be seen on stained thin blood films, but the number of trypanosomes in the circulation can vary considerably and is often low, so concentration techniques are usually required. Quantitative buffy coat method is the technique of choice for diagnosis of African sleeping sickness. This involves concentrating the trypanosomes at the plasma–platelet interface in a special microhaematocrit tube using differential centrifugation. Parasites are identified by labelling with the fluorescent marker acridine orange.

Wet preparations of fluid aspirated from the lymph nodes, bone marrow or cerebrospinal fluid (CSF) may also reveal live motile organisms. This technique is more likely to be productive in the case of infection with *T. brucei rhodesiense* than

T. brucei gambiense. The organisms are fragile, so care must be taken not to damage them when making the smears. The highly specific and sensitive serological card agglutination test for trypanosomiasis (CATT) may be used in conjunction with a direct visualisation method. If these tests are positive, then CSF examination is mandatory to determine the stage of the illness.

Haematological implications of treatment

Pentamidine and suramin are the drugs of choice for the early stages of West and East African trypanosomiasis, respectively. They have a cure rate of around 90% but are only able to achieve modest CSF concentrations so they cannot be used for later stages of the disease. The most common haematological side effects of pentamidine are leucopenia, thrombocytopenia and anaemia. Suramin has serious side effects, including haemolytic anaemia and bone marrow toxicity.

Melarsoprol, an arsenic-based compound, has been the drug of choice for late-stage sleeping sickness, but is highly toxic, with a mortality of 4–12%. Its main adverse effect is a fatal encephalopathic syndrome; haematological toxicity is not a problem. Eflornithine is expensive, but is of benefit in late-stage, particularly West African, disease; 25–50% of patients treated with this drug exhibit bone marrow toxicity with pancytopenia.

American trypanosomiasis (Chagas disease)

Epidemiology and biology

Chagas disease is caused by *T. cruzi*, which is transmitted by triatomine bugs that infest poor-quality housing. It can also be transmitted through blood transfusions and congenitally. It was restricted to a region in the Americas between Argentina and the southern states of the USA; however, because of increasing population mobility between Latin America and the rest of the world it is now detected in other parts of the United States of America, Canada, many European and some Western Pacific countries.

Clinical features

The incubation period is usually 7–14 days but may exceed 40 days if transmission is through blood transfusion. In the acute phase, swelling at the site of entry of the organism (chagoma) may be accompanied by fever, hepatosplenomegaly and lymphadenopathy. The trypanosomes multiply intracellularly in muscle, particularly the heart, colon and oesophagus. Once infection has occurred, the organisms will be present for life unless treatment is given. The chronic phase is associated with heart disease in 30% of cases, manifesting as arrhythmias and cardiomegaly. A small proportion of patients have involvement of the gastrointestinal tract and other hollow organs, resulting in loss of peristalsis, organomegaly and organ failure. Asymptomatic infection is common and poses a problem for blood transfusion services, so some countries routinely screen blood for American trypanosomiasis. Many cases do not become symptomatic until the chronic stage, which can occur 5–40 years after infection.

Diagnosis

Microscopy of blood smears can be helpful, but only in the acute phase of infection when parasites are circulating in blood. Sensitive serological tests based on enzyme immunoassay or immunofluorescence are therefore more commonly used as the primary diagnostic tool. Diagnosis is made based on at least two positive but different serologic tests. PCR may also be useful.

Haematological implications of treatment

Treatment of *T. cruzi* infection is with nifurtimox or benznidazole and if started early, the disease is curable. Major haematological side effects are uncommon with either drug, although agranulocytosis has been reported with benznidazole.

Leishmaniasis

Epidemiology and biology

Visceral and cutaneous leishmaniases are caused by protozoan flagellates that are transmitted through the infective bite of a phlebotomine sandfly. Leishmaniasis is second only to malaria as the most common parasitic cause of mortality. Following an infected bite, parasites spread from the inoculation site to the mononuclear phagocytic system. Only the visceral form (kala-azar) is associated with organisms in haemopoietic tissues and thus is considered here. Visceral leishmaniasis is due to the species *Leishmania donovani* and *L. infantum*. In 2013, it was endemic in 75 countries throughout the world, with extension limits from 45° N to 32° S. Over 90% of cases occur in Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan (Figure 51.7). A concomitant HIV infection increases the risk of developing active visceral leishmaniasis by between 100 and 2320 times. In southern Europe, up to 70% of cases of visceral leishmaniasis in adults are associated with HIV infection.

Clinical features

This depends on both the genotypic potential of the parasite and the immunological response of the patient. Incubation period varies from days to years but is generally 2–6 months. Onset can be sudden with high fever, or gradual with intermittent fever. Diarrhoea, joint pain, weight loss and bleeding gums occur in the acute phase. This is followed by progressive muscle wasting, fever, weight loss, anaemia and hepatosplenomegaly. The splenomegaly which appears early and worsens with the duration of the disease may reach into the left hypochondrium. In immunocompromised patients, such as transplant recipients and those with advanced HIV disease, kala-azar behaves like an opportunistic infection.

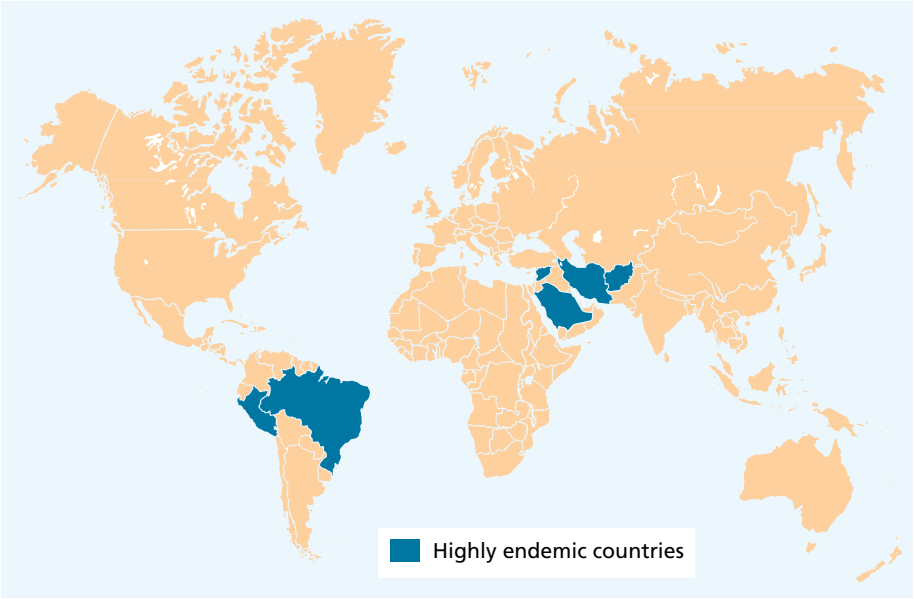


Figure 51.7 Global distribution of visceral leishmaniasis.

Haematological abnormalities

Normochromic normocytic anaemia is a frequent feature, and haemoglobin levels of 70–100 g/L are common. The massive splenomegaly results in hypersplenism with consequent pancytopenia. Liver dysfunction with jaundice, ascites and deranged coagulation may occur in the late stages and has a poor prognosis. The bleeding tendency may be exacerbated by thrombocytopenia. In all patients with unexplained splenomegaly, pancytopenia and fever, a high index of suspicion of leishmaniasis needs to be maintained to prevent fatalities.

Diagnosis

Definitive diagnosis is based on detection of the parasites, or their DNA, in smears of bone marrow, splenic aspirate or fluid aspirated from enlarged lymph nodes.

On microscopy, *Leishmania* are usually seen as intracellular amastigotes in mononuclear cells, but can also be seen extracellularly (Figure 51.8). They are 2–6 µm in diameter and contain a nucleus lying close to the rod-shaped kinetoplast, and an internal flagellum. Using a Romanowsky stain, the nucleus and kinetoplast stain purple and can be clearly distinguished. Amastigotes can be seen in both bone marrow aspirates and in trephine-impression smears. They are rarely seen in peripheral blood and then only in buffy coat preparations.

Microscopy is less sensitive than molecular diagnosis, particularly when there is coinfection with HIV. PCR can be performed on lesion aspirate, marrow, blood and biopsy material. The indirect fluorescent antibody tests ELISA and DAT are useful for detecting antibodies to visceral leishmaniasis, but results may be inconclusive in immunosuppressed patients.

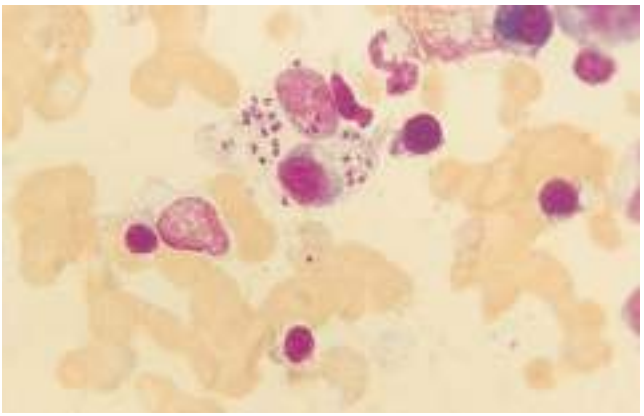


Figure 51.8 Bone marrow aspirate of leishmaniasis infection showing a macrophage containing numerous organisms. The presence of both a nucleus and a small paranuclear kinetoplast gives the organisms their characteristic 'double-dot' appearance (MCG × 940).

Haematological implications of treatment

For decades, the treatment of leishmaniasis has been based on pentavalent antimonials, although their mode of action is still unclear. Sodium stibogluconate is the most commonly used. It can be associated with worsening anaemia and thrombocytopenia, although its most detrimental effects are on cardiac function. Resistance levels to antimonials are high in countries such as India, Bangladesh, Brazil and Sudan, so other options including amphotericin, paromomycin and miltefosine need to be considered. HIV-coinfected patients do not respond well to antimonials, so for these individuals, amphotericin is the drug of choice. Amphotericin

commonly causes normocytic normochromic anaemia, but its most serious toxicity is related to renal, cardiac, neurological and hepatic dysfunction. Side effects can be reduced by using the liposomal preparation. Immunocompromised patients may require prophylaxis to prevent relapses.

Tropical diseases associated with changes in FBC and/or coagulation

Hookworm infection

Hookworms (*Ancylostoma duodenale*) and (*Necator americanus*) are common in the tropics and subtropics and affect 600 million people worldwide. They cause significant iron deficiency anaemia in all age groups, including 30–50% of anaemia in pregnancy. The hookworm eggs are generally transmitted between humans through contact with faeces. Most infections are asymptomatic, but chronic blood loss through the gut eventually leads to severe iron deficiency anaemia, and even cardiac failure. Other symptoms include itch, rash and abdominal discomfort. Eosinophilia is common and characteristic eggs can be seen on faecal microscopy. Albendazole or mebendazole are the usual treatments and iron deficiency should also be treated. Transfusion is rarely necessary and if it is required blood needs to be given very cautiously to avoid cardiac overload.

Schistosomiases (Bilharzia)

Schistosomiasis is a common intravascular infection caused by trematode worms acquired through contact with contaminated water. It occurs across Africa, Asia and South America. *Schistosoma. mansoni, intercalatum, japonica* and *mekongi* all cause intestinal schistosomiasis, whilst *S. haematobium* causes urinary schistosomiasis. There are three stages; acute (Katayama syndrome), chronic and advanced. Schistosomiasis is the second most common cause of iron deficiency worldwide after hookworm infection. It is due to blood loss from the urinary and gastrointestinal tracts. Eosinophilia occurs in 80% of cases. Anaemia of chronic disease, massive splenomegaly with hypersplenism, hepatomegaly and generalised lymphadenopathy can all occur. Iron deficiency should be treated and schistosomiasis treatment is with praziquantel, which has no significant haematological complications.

Viral haemorrhagic fevers

These are caused by arenaviruses, filoviruses, bunyaviruses and flaviviruses and are classified according to their reservoir hosts and their primary means of transmission. They are divided into:

- rodent-associated viruses (e.g. Lassa fever, hantaviruses);
- arthropod-borne viruses (e.g. dengue, yellow fever and Chikungunya viruses);
- unknown vectors or hosts (e.g. Marburg, Ebola).

They often occur in epidemics, have human-to-human transmission and, in non-tropical settings, may only be suspected if a relevant travel history is elicited from the patient. Dengue and yellow fever are becoming increasingly important imported infections.

Dengue

The four types of dengue virus belong to a group of flaviviruses and are transmitted by *Aedes* mosquitoes. Dengue is endemic in tropical areas of Asia, Africa, South America and the Caribbean and is particularly virulent in Southeast Asia, including Thailand and Vietnam. There has been a resurgence of the disease as a result of urbanisation, poverty and increasing travel. A recent study has shown that the introduction of the bacteria *Wolbachia pipientis* into *Anopheles aegypti* mosquitoes was effective in reducing the incidence of symptomatic dengue and hospitalisations.

Primary infection in young children is usually asymptomatic. Older children and adults develop acute fever, headache and myalgia ('breakbone fever'). Leucopenia may accompany this stage of the illness. Severe complications may arise in those who have had previous infection. These include hypotensive shock, bone marrow hypoplasia with neutropenia and abnormal megakaryopoiesis, leading to severe thrombocytopenia and spontaneous bleeding.

Yellow fever

Yellow fever virus is transmitted by *Aedes* mosquitoes and exists throughout equatorial Africa, northern and central southern America. Ninety percent of cases and deaths each year occur in Africa. Eliminate Yellow fever Epidemics (EYE) is a strategy that was launched in 2017 to support 40 at-risk countries in Africa and the Americas to prevent, detect and respond to suspected yellow fever cases and outbreaks. The partnership aims at protecting at-risk populations, preventing international spread and containing outbreaks rapidly.

The virus invades hepatocytes, causing hepatocellular dysfunction. After an incubation period of 3–4 days, fever, myalgia and back pain may be followed by jaundice, bleeding and, in severe cases, renal failure. Haematological changes include leucopenia with relative neutropenia, thrombocytopenia as part of a consumptive coagulopathy, initial haemoconcentration, and subsequent haemorrhage and haemodilution. Coagulation abnormalities include reduced fibrinogen and clotting factors II, V, VII, VIII, IX and X, with increased PT and APTT, as well as the presence of fibrin degradation products. In 85% of cases, there is spontaneous improvement, with the disappearance of symptoms in 3–4 days. The remaining 15% enter a toxic phase which has a 50% mortality.

Diagnosis can be difficult, as yellow fever (preventable by vaccination) can be confused with several febrile and haemorrhagic illnesses. Antibodies can be detected in the serum and treatment is symptomatic.

Lassa fever, Ebola virus and Marburg virus

These are endemic in equatorial Africa and are important because they cause potentially fatal infections and have the ability to spread from person to person. Only about 10% of infected individuals become ill. Of these 1–2% develop fatal disease. The clinical features of these three haemorrhagic fevers are similar and are characterised by headache, fever and oesophagitis. Spontaneous bleeding occurs in 25% of hospitalised patients and is thought to be due to increased vessel permeability and abnormal platelet function. Death is due to multi-organ failure and shock. Case reports suggest that treatment with ribavirin may be helpful and a promising vaccine against Ebola virus has recently been tested in clinical trials in west Africa. To date, there is no licenced vaccine against Ebola but one has been approved for compassionate use.

Non-specific haematological abnormalities associated with tropical diseases

Anaemia

Anaemia of chronic disease is a common and non-specific finding in many types of tropical infections and may be exacerbated by nutritional deficiencies. Some infections are responsible for specific types of anaemia. For example, hookworm (which is common in farmers who work barefoot on infected soil), and schistosoma infections are two of the most common causes of iron deficiency anaemia worldwide. For both conditions, a good clinical history and a thorough examination of a stool (for hookworms and schistosoma) or urine sample (for schistosoma) is important for diagnosis. Secondary megaloblastic anaemia can result from consumption of the human host's vitamin B12 when they are infected by the tapeworm *Diphyllobothrium latum*. Intraerythrocytic parasites, such as those that cause malaria and babesiosis, may be associated with significant intravascular haemolysis.

White cell abnormalities

Severe infections, particularly those due to bacteria, may cause a neutrophilia and a leukaemoid reaction with circulating myeloid precursors and neutrophils with toxic granulation, vacuolation and Döhle body formation. Lymphocytosis with neutropenia, splenomegaly, nose bleeds, rash and neurological complications can occur in rickettsial infections (e.g. typhus, Q fever and trench fever). Some of these, such as trench fever, may persist for many years and are transmissible in blood transfusions. The absence of neutrophilia in association with marked fever is a particular feature of typhoid. Lymphocytosis or monocytosis may also be present in typhoid; other clinical features include hepatosplenomegaly and, in severe disease, haemorrhage from ileal ulcers exacerbated by disseminated intravascular coagulation. Helminths and other predominantly tropical organisms that invade tissues may be associated with a significant eosinophilia ($>0.5\times10^9$/L). Such diseases include loaiasis, lymphatic filariasis, schistosomiasis, trichinosis, toxocariasis, strongyloidiasis, hydatid disease, oriental liver flukes and guinea worm.

Platelet abnormalities

Thrombocytosis may occur as a non-specific change associated with inflammatory conditions, including severe infections. Thrombocytopenia is usually secondary to hypersplenism (e.g. due to schistosomiasis, hyper-reactive malarial splenomegaly or disseminated intravascular coagulation). Thrombocytopenia can also occur in malaria, typically due to *P. falciparum*, either as a consequence of direct invasion of platelets by the parasite or by secondary mechanisms.

Hypersplenism

Hypersplenism is a clinical syndrome characterised by splenomegaly, with a reduction in the number of one or more types of blood cells. There is reduced survival of platelets and red cells and a normocellular or hypercellular bone marrow. Hypersplenism can result from any cause of splenomegaly, but it is most commonly associated with splenomegaly secondary to portal hypertension and haematological disorders. Tropical infections associated with massive splenomegaly (Figure 51.9) include hyper-reactive malarial splenomegaly, visceral leishmaniasis, schistosomiasis and trypanosomiasis. In the tropics, portal hypertension associated with schistosomiasis is one of the commonest cause of hypersplenism.

The cytopenias in hypersplenism are due to a combination of sequestration and haemodilution and the degree of plasma volume expansion is roughly proportional to the size of the spleen. The thrombocytopenia and neutropenia are rarely severe enough to cause clinical problems. The thrombocytopenia is primarily due to pooling of platelets in the spleen which, when massively

Figure 51.9 Patient with massive splenomegaly due to hyper-reactive malarial splenomegaly.

enlarged, can hold up to 90% of the platelet mass. Neutropenia is the result of increased marginalisation of granulocytes. Treatment of the underlying disorder generally leads to regression of the splenomegaly, with resolution of the haematological abnormalities. Occasionally, splenectomy is necessary, but this should only be undertaken after a careful assessment of the risks and benefits, and adherence to recommendations (e.g. vaccinations, antibiotic prophylaxis) regarding infection prevention.

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CHAPTER 52

Lysosomal storage disorders

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Lysosomal storage disorders

Lysosomes are membrane-bound intracellular organelles which function as the major degradative compartment of mammalian cells. They are morphologically heterogeneous and are distinguished from other organelles by the presence of catalytic enzymes and lysosome-associated membrane proteins (LAMPs). More than 50 lysosomal hydrolases, active at acidic pH, catalyse the degradation of macromolecules (lipids, proteins, nucleic acids, glycosaminoglycans and oligosaccharides). These include breakdown products of blood cell membranes, gangliosides from neurons and glycosaminoglycans derived from connective tissue and extracellular matrix as well as substrate of intracellular origin. Substrate is delivered to the lysosome by endocytosis, pinocytosis, phagocytosis or autophagocytosis, or imported from the cytoplasm using biological chaperones. Apart from its role in the disposal and recycling of cellular waste, the lysosome is currently viewed as a dynamic regulatory hub for several cellular functions, such as cholesterol handling and adaptation to nutrient status and environmental stimuli.

In most lysosomal storage disorders (LSD), an inherited deficiency of a specific lysosomal enzyme results in the accumulation of undegraded substrates within the lysosome. In others, deficiency or malfunction of activator or transport proteins results in substrate accumulation. Individual mutations of relevant genes give rise to variable levels of residual enzyme activity, one of the determinants of phenotypic severity. The resulting diseases are categorised according to the major accumulated substrate, for example the mucopolysaccharidoses, sphingolipidoses and glycoproteinoses. Whilst different lysosomal storage diseases have the characteristic organ distribution patterns of the stored metabolites, clinical manifestations often extend beyond this and there can be considerable interindividual heterogeneity. Genotype–phenotype relationships are often unclear, and siblings can have disparate clinical manifestations, suggesting the modifying effect of genetic and environmental factors on presentation and severity.

The pathophysiology of lysosomal disorders results both from the disruptive effect of storage on cellular organisation and membrane functions including signal transduction and ion transport and also on direct stimulatory effects of the substrate molecule. This may result in immune stimulation, cytokine secretion, cellular proliferation, disturbed calcium homeostasis, exaggerated inflammation and perturbed control of apoptosis.

Prevalence

The prevalence of LSDs ranges from 1 in 57,000 for Gaucher disease to as low as 1 in 4.2 million for sialidosis. Recent screening studies suggest that the genetic prevalence may be higher; for example approximately 1:2500 for Fabry disease with under detection due to minimally symptomatic forms and lack of awareness or misdiagnosis of multisystem conditions. Although the diseases are individually rare, their combined overall birth prevalence is 1 in 5000–8000.

Diagnosis

The diagnosis of LSDs requires a high degree of clinical suspicion. Storage material may be discovered incidentally within biopsy specimens taken to investigate unexplained symptoms; e.g. a bone

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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marrow for the investigation of cytopenias in Gaucher disease or kidney biopsy for unexplained renal dysfunction in Fabry disease. A number of LSDs may present with hepatosplenomegaly or other haematological manifestations (Table 52.1).

The identification of substrate deposits in tissue biopsy is subject to a differential including other LSDs and pseudo forms due to high cellular turnover in unrelated conditions or lysosomal effects of some drugs (Table 52.2); and thus, requires the measurement of lysosomal enzymes or identification of a mutation to confirm the specific diagnosis. Substrate levels may also be measured in plasma or urine and may be used as biomarkers of severity or response to therapy but are rarely used in isolation as diagnostic tests.

Activity of lysosomal enzymes may be measured in plasma or leucocytes using commercially available synthetic or naturally occurring labelled substrates. Subsequent identification of a specific mutation in the corresponding gene may facilitate prognostication and rapid screening of other family members.

Early or pre-symptomatic diagnosis through cascade screening has been advocated to optimise the possibility of monitoring and early intervention with specific therapies. Analysis of blood (blood spot on filter paper) or urine for enzymes or substrate-derived metabolites has facilitated high throughput screening for LSDs, and newborn screening for specific conditions is now available routinely in some areas.

Therapy

Specific treatment of lysosomal storage disorders involves reduction of the stored compound and prevention of its re-accumulation. This has been achieved by elevation of enzyme activity through stem cell transplantation, infusion of the missing enzyme (enzyme replacement therapy or ERT) and gene therapy or stabilisation of protein folding variants by pharmacological chaperones. Conversely, reduction of the substrate can also be achieved by limiting its synthesis through inhibition of anabolic enzymes, so-called substrate reduction therapy (SRT).

Stem cell transplantation

Patients with Gaucher disease, metachromatic leucodystrophy, Krabbe disease and MPS type I have undergone stem cell transplantation. Donation of stem cells has largely been from HLA-identical siblings, but as the highest level of enzyme activity will be supplied by donor cells from non-carriers, cord blood from unrelated donors has recently been used with good results. In LSDs without a neurological component, other forms of treatment have largely superseded stem cell transplantation.

Table 52.1 Haematological presentations of Lysosomal storage disorders.

	Blood cells	Plasma	Bone Marrow	Liver and spleen	Abnormalities of coagulation
Gaucher disease	Cytopenias	Mono clonal gammopathy Elevated markers of macrophage activation	Gaucher macrophages	Hepatosplenomegaly	Platelet dysfunction Coagulation factor deficiencies possibly due to consumption
Pompe Disease	PAS-positive glycogen granules in lymphocytes			Hepatomegaly in infantile Pompe disease	
CESD/ Wolmans	Cytopenias related to hypersplenism		Features of secondary HLH	Hepatomegaly and in some cases splenomegaly	
MPS	Cytopenias related to hypersplenism			Hepatosplenomegaly	
Fabry	Renal anaemia		Lipid laden macrophages		
Niemann Pick	Cytopenias related to hypersplenism		Sea Blue histiocytes (NPB) Lipid-laden macrophages NPC	Hepatosplenomegaly	

Table 52.2 Examples of differential diagnosis of lipid-laden macrophages, pseudo-Gaucher cells and drug-related lysosomal storage.

Differential diagnosis of Foam Cells	Causes of pseudo Gaucher cells	Drugs
Niemann–Pick Disease	Chronic myeloid leukaemia	Amiodarone
Sea Blue histiocytosis	Multiple myeloma	Chloroquine
Obesity	Thalassaemia	Prolonged liposomal amphotericin
Multiple sclerosis	Haematological proliferative disorders	Parenteral nutrition with fat emulsion
Batten disease	Mycobacterial infections affecting the bone marrow	
Takayasu's arteritis		

Enzyme replacement therapy

Intravenous ERT is now standard therapy for a number of LSDs. Recombinant human enzyme can be produced in transduced eukaryotic cell lines, using Chinese hamster ovary (CHO) cells or plant (e.g. carrot or tobacco) cells. Gene activation technologies have been applied to cultured human fibroblasts for the manufacture of lysosomal enzymes. The effectiveness of ERT depends on the accessibility of the site of the pathology to exogenous enzyme and the ability of affected cells to internalise the enzyme. ERT is effective in reducing the non-neuronopathic manifestations of a number of disorders, including Gaucher disease type I, Fabry disease, Pompe disease and MPS types I, II, IVA, VI and VII and Lysosomal acid lipase deficiency (Wolman disease/Cholesterol Ester Storage Disorder). Infused enzymes do not cross the blood–brain barrier and ERT does not prevent the onset and progression of neurological symptoms. Intrathecal administration is under investigation in neurological forms of MPS I and II. Intraventricular infusion of cerliponase alfa in patients with ceroid lipofuscinosis type 2 (CLN2) has been shown to modify disease course; although early trials were hampered by adverse events including failure of the intraventricular device and device-related infections. Anti-Drug Antibodies may develop, especially in the absence of endogenous enzyme (Cross-reactive immunological material or CRIM negativity) and this may result in infusion reactions and attenuate the effectiveness of the enzyme.

Substrate reduction therapy

Analogues of small molecules are used to inhibit the activity of synthetic enzymes, thereby decreasing the rate of substrate accumulation. For example, ceramide analogues can be used to reduce the activity of the anabolic enzyme glucosylceramide synthetase. SRT is available for Gaucher disease and under clinical investigation in Fabry disease The small molecules used in SRT can be given orally, are easily absorbed and have a wide tissue distribution. SRT may also potentially have therapeutic utility in crossing the blood– brain barrier and studies are currently underway in the investigation of SRT for the neurological manifestations of type III Gaucher disease and a late-onset form of Tay–Sachs disease.

Pharmacological chaperone therapy

Some mutations lead to reduced activity of a lysosomal enzyme by causing misfolding or abnormal intracellular transport. The oral administration of a small molecule (pharmacologic chaperone) may improve enzyme activity by binding specifically to mutant peptides, aiding their passage through the endoplasmic reticulum and rescuing them from proteasomal degradation; ultimately guiding the synthesised enzyme to the lysosome. The agent dissociates from the enzyme within the acidic environment of the lysosome. Of note, pharmacological chaperones are only effective where the individual mutation results in a protein which is 'amenable' to binding of the chaperone and enhancement of residual enzyme activity; therefore, it can be viewed as an example of a personalised approach to therapy. Pharmacological chaperone therapy is available for Fabry disease where approximately 40–50% mutations are 'amenable' to the agent.

Genetic therapies

Agents that bind to ribosomes (e.g. derivatives of gentamicin) and which modify the processing of RNA transcripts to allow 'read-through' of premature stop codon mutations are another class of mutation-specific therapy currently being evaluated in lysosomal storage disorders. Independently, gene therapy is under investigation for Fabry and Gaucher, Pompe and the MPS disorders. In the case of ex vivo gene therapy, autologous stem cells are transduced with a virus (e.g. lentivirus) containing the gene of interest and after conditioning chemotherapy the modified cells are given back to the patient. Stem cells populate various organs, and synthesise and secrete enzyme into the local environment. In vivo, gene therapy requires a viral vector to insert copies of the gene of interest into target cells. For example, in the case of adenoviral vectors hepatocytes express the therapeutic transgene and the enzyme is secreted from liver into the circulation from where it is taken into relevant cells and organs. Additional investigational approaches include mRNA therapy (wherein the enzyme transcript is encapsulated within a lipid nanoparticle and directed to the liver) and gene editing involving the CRISPR system.

Prognosis

The clinical course of infants diagnosed with an LSD associated with central neurological involvement usually follows a predictable path of loss of learned skills and neurological deterioration until premature death results from infection and progressive organ damage. When onset is later, in adolescence or adulthood, the clinical course is more varied, and the prognosis depends on the major organ systems affected.

Gaucher disease

Gaucher disease is an autosomal recessive condition resulting from mutation within the *GBA* gene and deficiency of the enzyme β glucocerebrosidase, a lysosomal enzyme that hydrolyses glucosylceramide to glucose and ceramide (Figure 52.1). The substrate, glucosylceramide, accumulates in lysosomes of both reticuloendothelial cells and cells outside of the reticuloendothelial system such as immune cells and osteoblasts. It is one of the commonest LSDs, with an estimated incidence of 1 in 60,000 to 1 in 80,000 individuals.

Clinical features

Three main clinical phenotypes are observed (Table 52.3), determined in large part by the residual activity of the mutant enzyme. All three types are progressive disorders. Enzyme activity in type II is so low that abnormal cells accumulate in the central nervous system (CNS). This is the acute neuronopathic form of the disease, which presents with neurological complications in early

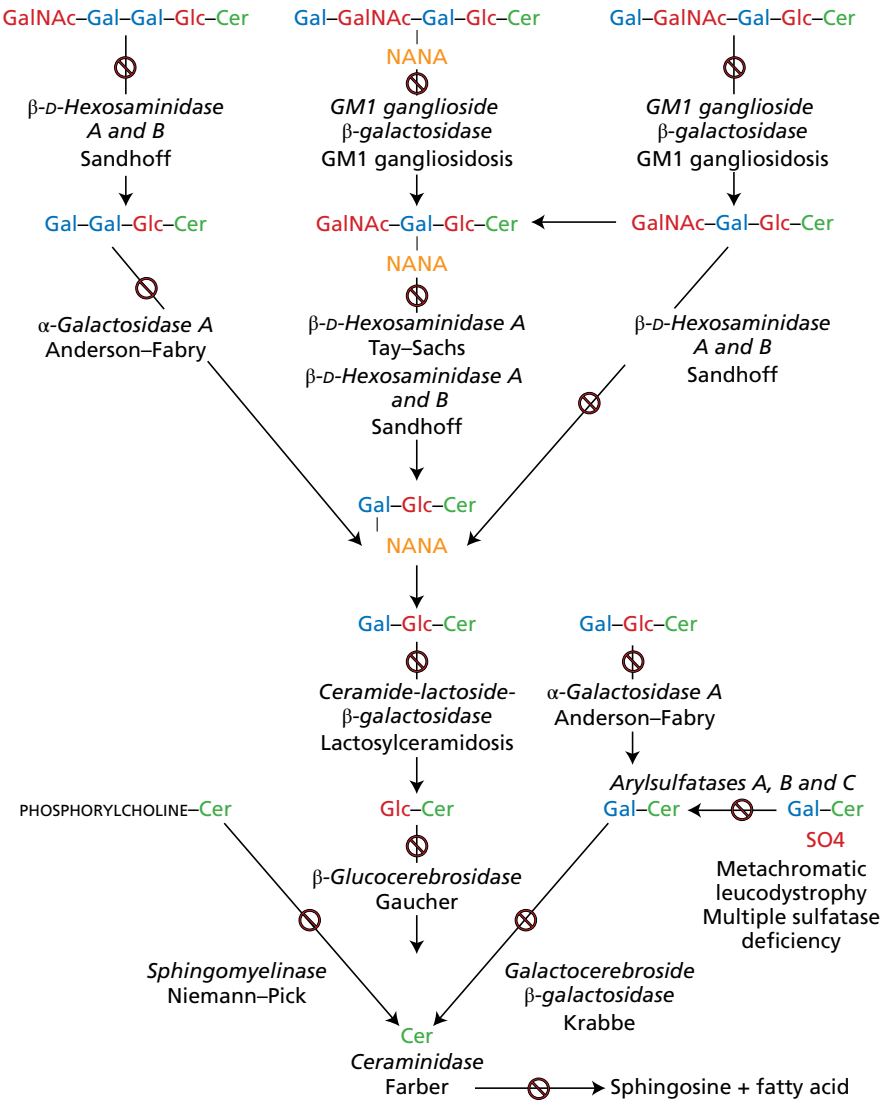

Figure 52.1 Biochemistry of lysosomal storage disorders.

Table 52.3 Clinical manifestations of Gaucher disease.

Manifestation	Type I	Type II	Type III
Onset	1 year	<1 year	2–20 years
Hepatosplenomegaly	++	+/−	+
Bone disease	++	−	+/−
Cardiac valve disease	−	−	+
CNS disease	−	+++	+/−
Oculomotor apraxia	−	+	+/−
Corneal opacities	−	+/−	+/−
Age at death	60–90 years	<5 years	<60 years

infancy and usually leads to death before the age of 2 years. Type III disease is the subacute neuronopathic form that leads to a slowly progressive neurodegenerative disorder.

Type I is the commonest form of Gaucher disease and typically does not cause neurological disease. It is particularly prevalent amongst subjects of Ashkenazi Jewish origin; where as many as 1 in 15–20 subjects are carriers, and approximately 1 in 800–1000 subjects are affected (i.e., homozygous or compound heterozygotes). Type I Gaucher disease is a heterogeneous disorder that may present in childhood through to late adult life. It is likely that many undiagnosed subjects are asymptomatic. Tissue damage due to accumulation of sphingolipid-laden macrophages in reticuloendothelial organs causes hepatosplenomegaly, skeletal disease and bone marrow infiltration, leading to cytopenias. Bone disease includes osteonecrosis, osteosclerosis, reduced bone density and in some cases pathological fractures. Rarer manifestations of type I Gaucher disease include lymphadenopathy, pulmonary disease, skin involvement and peripheral neuropathy. Patients with type I Gaucher disease have an increased incidence of malignancy generally and an increased incidence of haematological malignancies, especially B-lymphocyte disorders (myeloma, monoclonal gammopathy of undetermined significance) and myelodysplasia. Attribution of bone pathology to myeloma may be difficult in the context of Gaucher-related bone disease and care should be taken in monitoring the progression to myeloma amongst patients with a monoclonal gammopathy. Also, there is an increased incidence of Parkinson disease amongst affected individuals and carriers. The underlying mechanism is unknown, but experimental and animal model studies suggest that either diminished activity or misfolding of glucocerebrosidase may lead to excess deposition of α-synuclein in the basal ganglia, which is a pathological hallmark of Parkinson disease.

Laboratory features

More than 300 different mutations have been described in *GBA1*. The commonest mutation causing type I disease is a single base-pair substitution in codon p.Asn409Ser (formerly known as p.N370S), which accounts for approximately 70% of mutant alleles in affected Ashkenazi Jewish subjects. A base-pair substitution in codon p.Leu483Pro (formerly known as p.L444P) is the most frequent mutation underlying neuronopathic Gaucher disease. Diagnosis is confirmed by enzymatic assay of β-glucocerebrosidase activity in leucocytes or fibroblasts. Enzyme activity does not always identify heterozygote subjects and measured enzyme activity correlates poorly with clinical severity.

Splenic enlargement and marrow infiltration frequently lead to anaemia, thrombocytopenia and leucopenia. Polyclonal gammaglobulinaemia is found in more than one-third of patients, and monoclonal gammopathy of undetermined significance (MGUS) is seen in up to 20%. Liver function tests are often abnormal, reflecting infiltration of the liver by Gaucher cells leading to necrosis, fibrosis and, occasionally, cirrhosis. There is an increased incidence of gallstones. The serum cholesterol level is typically lowered. Gaucher disease is associated with a bleeding diathesis, attributable to abnormal platelet function and thrombocytopenia. Factor XI deficiency is commonly observed and is largely due to co-inheritance of other genetic abnormalities that are also common amongst Ashkenazi Jews.

The abnormal lipid-laden macrophages are readily detected on tissue biopsy (e.g. bone marrow aspirate; Figure 52.2), although biopsy is no longer considered necessary to make the diagnosis. The enzyme chitotriosidase is derived from macrophages and is typically grossly elevated in untreated Gaucher disease; it declines progressively with treatment.

However, up to 6% of the population are chitotriosidase deficient because of a 24-bp duplication in the encoding gene and in this case Pulmonary activation-regulated cytokine (PARC, CCL18), which is also elevated in plasma of patients with Gaucher disease may be useful for monitoring. PARC is a member of the CC chemokine family and its over-expression may be relevant to some of the pathophysiological features of Gaucher disease, such as abnormalities in neutrophil chemotaxis. Biomarkers of macrophage activation are complemented by measurement of

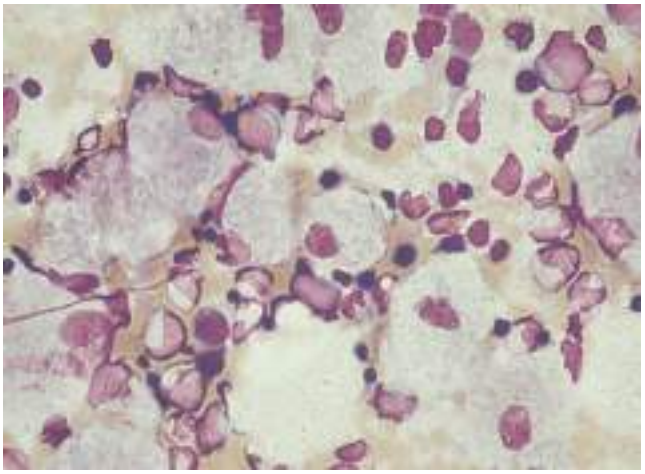

Figure 52.2 Gaucher cells in the bone marrow.

substrate-related metabolites in plasma. In particular, the level of the deacetylated derivative of glucosylceramide, glucosyl sphingosine (Lyso-GL1) has been demonstrated to correspond well to overall disease burden, mutation and response to therapy.

The serum levels of ferritin, angiotensin-converting enzyme (ACE), tartrate-resistant acid phosphatase (TRAP) and transcobalamin 2 are also typically elevated in Gaucher disease, and a wide variety of enzymes, cytokines and coagulation factors are perturbed. Interleukin (IL)-1, IL-6, IL-10 and tumour necrosis factor (TNF) have been implicated in the pathogenesis of Gaucher disease. Elevated serum levels of IL-6, which is produced by macrophages, endothelial cells, fibroblasts and T cells, have been found to correlate with an index of severity in patients with Gaucher disease and may be instrumental in the pathogenesis of B-cell dysregulation and bone disease. IL10, which in general inhibits the synthesis of other inflammatory cytokines, is also elevated; and may represent an abnormal state of immune activation. Whilst providing insights into the pathogenesis of Gaucher disease, these molecules are not routinely measured in the management of patients with Gaucher disease.

Treatment

Patients with Gaucher disease should be evaluated by an experienced physician. Gaucher disease is the first lysosomal storage disorder to be treated with ERT, and a number of different ERT preparations are now available for the treatment of type I Gaucher disease (Cerezyme Sanofi Genzyme; VPRIV Takeda; Taliglucerase Pfizer Protalix). Biosimilar ERT preparations are available commercially in some areas. Indications for ERT include significant cytopenias (e.g. Hb < 100 g/L, platelets < 100×10^9/L), skeletal disease and significant hepatosplenomegaly. ERT has no discernible impact on CNS disease; type II Gaucher disease is therefore unresponsive to ERT and ERT is indicated for the systemic, but not the CNS, manifestations of type III.

ERT is administered by intravenous infusion (often in the patient's home) at a dose of up to 60 units per kg every 2 weeks. The dose of therapy is titrated against the severity of clinical and laboratory changes and may be gradually lowered as the disease burden declines. Patients with less advanced disease may require lower doses, and some patients may eventually only require monthly infusions. A small proportion of patients (<10%) develop antibodies, but these are not usually neutralising and do not affect treatment efficacy. Infusion reactions are rare and easily managed. Patients are monitored regularly in specialist units with blood tests and evaluation of bone response by MRI of the skeletal system and bone densitometry.

Two forms of oral SRT are approved for Gaucher disease. Miglustat (Actelion/Johnson and Johnson) is licenced for mild to moderate type I disease where ERT is not possible; reported side effects include gastrointestinal disturbance and peripheral neuropathy. Eliglustat (Sanofi Genzyme) is a ceramide analogue that has more recently been developed as SRT. It is approved for use in naive or ERT-experienced patients. It is metabolised through CYP2D6 and determination of metaboliser status of patients is required for correct dosing and prediction of potential drug-drug interactions. Allogeneic stem cell transplantation is curative, although not without risk. Gene therapy is currently under investigation.

Supportive therapy is frequently required. The skeletal disease (Figure 52.3) is painful and patients may require analgesia. Regular orthopaedic assessments are advised. Prior to the use of ERT, patients frequently developed acute 'bone crisis' – episodic severe pain, typically in the limbs and often precipitated by fever and dehydration. Blood component therapy may be required for cytopenic patients, particularly at the time of orthopaedic or other surgical intervention. Splenectomy should be avoided if possible as splenectomised subjects are more likely to develop tissue infiltration in other organs (e.g. liver, lungs and skeleton).

Figure 52.3 MRI scan showing skeletal changes in Gaucher disease.

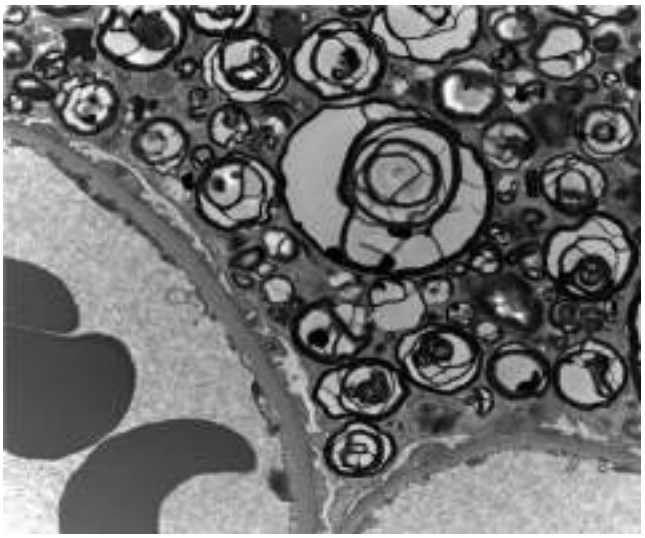

Figure 52.4 Renal biopsy in Fabry disease showing accumulation of inclusions on electron microscopy.

Fabry disease

Fabry disease is an X-linked lysosomal storage disorder due to mutation within the gene for α-galactosidase A (*GLA*) (see Figure 52.1). The resulting inability to catabolise glycosphingolipids leads to progressive accumulation of the substrate globotriasylceramide and its analogues including globotriaosylsphingosine (Lyso GL3) in tissues. In contrast to Gaucher disease, the lipid accumulation in Fabry disease affects a range of cells (e.g. endothelial cells, epithelial cells, myocytes) within a broad distribution of tissues and organs, particularly the kidneys (leading to renal failure), heart (causing ventricular hypertrophy and conduction disturbances) and vasculature of the CNS (increasing the risk for stroke). It is panethnic and whilst the incidence has been reported to be 1:100,000 it is likely to be underdiagnosed and milder variants may be much commoner and may present as left ventricular hypertrophy, stroke/transient ischaemic attack and renal failure. Although it is X-linked, Fabry disease typically causes manifestations in females, though these occur later and are usually less severe than in males. Diagnosis is by assay of α-galactosidase A activity in leucocytes and detection of molecular abnormalities within the *GLA* gene. Diagnosis may be suggested by skin, cardiac or renal (Figure 52.4) biopsy but should be confirmed by enzyme and DNA analysis.

Clinical features and treatment

A skin rash (angiokeratoma) and burning pain in limbs (acroparaesthesia) are early symptoms (under 10 years old). In late childhood, reduced sweating, abdominal symptoms and lymphoedema are characteristic. Renal failure, cardiac failure, stroke and CNS organ involvement are later features. When untreated, life expectancy in the classical form of the condition is 40–50 years for men and 50–65 years for most women.

ERT for Fabry disease has been available since 2001. Three formulations are available: a recombinant galactosidase that is generated in CHO cells (agalsidase β, Sanofi Genzyme Corporation, MA, USA); and an enzyme produced in a human fibroblast cell line (agalsidase α, Takeda), and a pegylated enzyme produced in tobacco cells (pegunigalsidase alfa Chiesi, Parma, Italy). Beneficial clinical effects have been observed in renal and cardiac function, pain, hearing loss and gastrointestinal symptoms. More recently, an oral pharmacological chaperone (migalastat, Amicus Therapeutics New Jersey USA) has been approved for patients with Fabry disease and amenable mutations. The drug is taken every other day and has been demonstrated to have beneficial effects on disease substrate, left ventricular mass and Fabry-related clinical events. Patients in phase III clinical studies exhibited stability of renal function. Substrate reduction therapies and gene therapy are currently in development.

Pompe disease

Pompe disease (glycogen storage disease type II) is caused by deficiency of acid α-glucosidase. It is characterised clinically by an early infantile subtype associated with cardiomyopathy, hypotonia and reduced survival and a later-onset form with features consistent with a limb-girdle muscle disease phenotype and survival into adulthood. On blood film examination, lymphocytes show glycogen vacuolation, which is positive on PAS staining. The prevalence of Pompe disease has been estimated to be 1 in 146,000. In untreated patients with the late-onset form of Pompe disease, muscle strength and pulmonary function deteriorate over time.

Alglucosidase-α (Myozyme) is a recombinant formulation of human acid α-glucosidase generated from the transduction of CHO cells. In infantile-onset patients, enzyme therapy has been shown to extend overall and ventilator-free survival and improve lung function and mobility in adults with later onset form of Pompe. Two novel intravenous enzyme replacement therapies, one in combination with an oral pharmacological chaperone have recently become available, and gene therapy is in clinical phase development.

Niemann–Pick disease

Niemann–Pick disease is divided into subtypes A and B resulting from sphingomyelinase deficiency and subtype C resulting from defects in cellular cholesterol trafficking. NPC can arise

from mutations in one of two non-catalytic proteins: NPC1 a lysosomal transmembrane protein, and NPC2 which is an intralysosomal soluble protein. Patients with type A exhibit neurodegenerative disease resulting in death in infancy, those with type B have lung, but not neurological involvement, and those with type C show slowly progressive neurological disturbance. Hepatosplenomegaly may be found in all three types. Over 100 sphingomyelinase mutations causing Niemann–Pick disease type A or B have been described, and DNA-based carrier screening has been implemented in the Ashkenazi Jewish community. A clinical trial of ERT (olipudase alfa) has demonstrated safety and efficacy in treating adult patients with non-neurological sphingomyelinase-deficient Niemann–Pick disease. The use of inhibitors of glycolipid synthesis, including miglustat, appears promising in the therapy of Niemann–Pick disease type C. Two other options: 2-hydroxypropyl-β-cyclodextrin A (a solubilising agent of lipophilic compounds) and arimoclomol (which amplifies the heat shock response to target NPC protein misfolding), are currently also under investigation for their effectiveness in treating NPC.

MPS disorders

In individuals with MPS disorders, deficiency or malfunction of specific lysosomal enzymes leads to accumulation of mucopolysaccharides (glycosaminoglycans) in the bone, cartilage, tendons, corneas, skin and connective tissue; affecting the respiratory system, liver, spleen, central nervous system and bone marrow. There are several different types of mucopolysaccharidosis and with the exception of MPS II which is X-linked, are mainly autosomal recessive traits. Affected individuals may have normal intellect, or in the case of more severely affected profound intellectual disability, developmental delay or severe behavioural problems including hyperactivity. Coarse facial features, thickened skin, corneal clouding, short stature skeletal irregularities and hepatosplenomegaly are amongst the shared clinical features. People with MPS also have a significantly shortened life span. ERT is available for MPS I, MPS II, MPS IVA, MPS VI and MPS VII, and in development for other disorders along with gene therapy. Bone marrow transplantation has been used with variable effect.

Cholesteryl ester storage disease and Wolman's disease

Cholesteryl ester storage disease (CESD) results from deficiency of the lysosomal acid lipase (LAL) enzyme. Mutations in the encoding *LIPA* gene that are associated with residual enzyme activity cause CESD, whereas mutations that abrogate enzyme activity cause Wolman disease. In CESD, patients present with hypercholesterolemia, hypertriglyceridemia, HDL deficiency, abnormal lipid deposition in many organs, hepatomegaly and in some cases splenomegaly. Liver involvement may progress to cirrhosis. Wolman disease manifests in childhood with adrenal calcifications, abdominal distention, vomiting and significant hepatosplenomegaly. Therapeutic tools include a hypolipidaemic diet and statins, and where indicated hepatic transplantation. ERT with sebalipase alfa (Kanuma, Alexion Pharmaceuticals Inc) is available.

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CHAPTER 53

Obstetric haematology

53

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Introduction

Obstetric haematology concerns the care of women before, during and after pregnancy with primary or secondary haematological disease. This is a high-stakes area of medicine where careful planning involving the multidisciplinary team and attention to detail can make the difference between life and death of a young woman and/or her baby. Normal pregnancy is associated with substantial physiological changes to meet the needs of the developing fetus but these changes, particularly if unbalanced, can themselves lead to complications. For example, the massive increase in uterine blood flow and vascular compliance necessary to maintain blood supply to the fetus can lead to significant haemorrhage at the time of placental separation. Changes in coagulation factors help to mitigate this risk but increase the potential for systemic thromboembolic events. The increased demand for iron and other nutrients by the growing fetus frequently results in maternal deficiencies and close proximity of the fetal and maternal circulations enables an effective transfer of nutrients and oxygen but exposes the mother to fetal antigenic material with potential alloimmune sensitisation. Although most pregnancies progress without problem, knowledge and awareness in this field of medicine are critical to prevent avoidable complications.

Cellular changes during pregnancy

Physiological changes in haematological parameters occur during normal pregnancy and in the puerperium. It is important to understand these changes to permit accurate interpretation of investigation results and avoid over-diagnosing abnormality.

Red cells

Plasma volume increases by up to 50% during pregnancy to accommodate the requirements of the developing fetus. This increase exceeds the 20–30% corresponding increase in red cell mass and results in a decrease in haemoglobin concentration. This physiological or 'dilutional' anaemia begins towards the end of the first trimester and is maximal at 28–30 weeks gestation. Red cell count and haematocrit are correspondingly lower in pregnancy. Mean cell volume (MCV) increases by around 6fl but mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) remain unchanged.

The World Health Organisation and British Society for Haematology define anaemia in pregnancy as;

- Hb <110 g/L in first trimester
- Hb <105 g/L in second and third trimester
- Hb <100 g/L postpartum

White cells

Total white cell count is increased during pregnancy due to over 50% rise in neutrophils, which begins in the first trimester. An increased number of band neutrophils are seen with occasional more immature forms such as metamyelocytes or myelocytes. Toxic granulation is common. Lymphocytes are reduced by around one third. There is a monocytosis which is thought to help modulate the maternal immune response and prevent fetal rejection. Basophils and eosinophils do not change. The white cell count further increases during labour, delivery and the

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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postpartum period, returning to pre-delivery levels by 7 days postpartum and to pre-pregnancy levels by around 3 weeks.

Platelets

The platelet count falls during normal pregnancy with levels in the third trimester being approximately 10% lower than pre-pregnancy levels. The exact mechanism is unknown but thought to be a combination of haemodilution, increased consumption and pooling of platelets in the placenta. Thrombocytopenia outside of pregnancy is defined as a platelet count $<150 \times 10^9$/L; however, the lower limit of the normal range during pregnancy has been suggested to be 115×10^9/L. Thrombocytopenia is the second most common haematological abnormality seen during pregnancy after anaemia, with prevalence at the end of pregnancy between 6.6 and 11.6%. Severe thrombocytopenia is far less common with only 1% of healthy women having a platelet count $<100 \times 10^9$/L.

Anaemia

Iron deficiency

Iron deficiency is by far the most common cause of maternal anaemia and has substantial health implications for mother and baby as iron is needed for every cell in the body. Complications include fatigue, cardiovascular strain, cognitive and immune dysfunction, increased likelihood of blood transfusion, low birth weight, preterm birth, postpartum haemorrhage, postnatal depression and increased maternal and perinatal mortality.

UK guidelines are in place to aid prompt diagnosis and appropriate treatment. Haemoglobin is routinely checked in early pregnancy and at 28 weeks' gestation. If anaemia is diagnosed a trial of oral iron should be initiated without delay and used as a diagnostic tool, with assessment for Hb response 2 weeks later. Further investigation is only necessary if the Hb fails to show improvement after 2 weeks, in which case serum ferritin and folate should be checked in the first instance. Serum ferritin is also required in women with known haemoglobinopathy to exclude an iron-loading state before treatment is given.

Non-anaemic iron depletion is also important to treat to avoid the complications of iron deficiency as well as the development of anaemia. Risk factors for iron deficiency should be considered, such as previous anaemia, history of bleeding or blood donation, an interpregnancy interval less than 1 year, multiple pregnancy and multiparity. These women should either be offered oral iron empirically or have their serum ferritin checked to confirm iron depletion.

Whilst all women should receive dietary advice, to optimise iron intake, this is inadequate as sole treatment for established iron deficiency and iron supplementation is necessary. Tablets containing 60 mg elemental iron are ideal and tolerance is significantly improved by correct administration. This includes taking tablets with water or orange juice on an empty stomach an hour before food, drink, multivitamins and other medications, all of which interfere with iron absorption. Tablets should be preferably taken early in the morning when hepcidin levels are lowest. Optimal value from each tablet is gained by daily, or alternate day, administration, to allow time for the increase in hepcidin levels to return to baseline.

Intravenous iron should be offered to anaemic women in the second trimester onwards who are intolerant of or unresponsive to oral iron and women presenting after 34 weeks' gestation with confirmed iron deficiency anaemia and Hb <100 g/L where there is insufficient remaining time before delivery for correction of anaemia with oral iron.

Women at risk of postpartum anaemia for example due to blood loss of more than 500 mL or uncorrected antenatal anaemia should have their Hb checked within 48 hours of delivery. If anaemia is confirmed, iron replacement with oral or intravenous iron should be initiated. Blood transfusion may be required if Hb is less than 70 g/L and there is cardiovascular compromise or significant risk of re-bleeding.

Folate deficiency

Women are advised to take 400 mcg/day of folic acid before conception until 12 weeks of pregnancy for the prevention of neural tube defects. Beyond 12 weeks, women remain at risk of folate deficiency and consequent anaemia as there is a significant increase in folate requirements, due to the increased cell replication that takes place in the fetus, uterus and bone marrow. Plasma folate concentrations decrease throughout pregnancy, reaching to half the non-pregnant levels by term.

Maternal anaemia due to folate deficiency may be difficult to diagnose. Coexisting iron deficiency can mask a macrocytosis and red cell indices are not necessarily helpful. Clinical suspicion is important; a diet containing low amounts of legumes and leafy green vegetables, malabsorption (most commonly coeliac disease), hyperemesis gravidarum and multiparity should prompt a test for serum folate. If anaemia is not responding to compliant iron replacement, folate deficiency should be considered. If found to be low, treatment is with oral folic acid 5 mg/day.

Folate requirements are increased in conditions associated with peripheral red cell destruction, such as sickle cell disease and other haemolytic anaemias, as well as with certain medications including antiepileptics. Prophylactic folic acid at 5 mg/day should be prescribed for women with these conditions.

Vitamin B12 deficiency

Vitamin B12 deficiency is uncommon during the reproductive years and as it can be associated with infertility, it is a rare finding in pregnancy. Serum vitamin B12 levels variably drop during pregnancy due to a fall in the inactive component of B12,

holohaptocorrin, and low levels rarely indicate true deficiency. Clinical assessment is therefore important, taking into account relevant symptoms such as paraesthesia and glossitis, along with consideration of risk factors such as a vegan diet, anorexia, gastrointestinal disease or surgery, and a personal or family history of autoimmune disease such as vitiligo, hypothyroidism and pernicious anaemia. The active B12 component, holotranscobalamin, can be assayed in some laboratories. Management of true B12 deficiency is with oral cyanocobalamin or intramuscular hydroxycobalamin depending on the likely cause.

Congenital anaemias

Inherited defects in red cell structure, and abnormal haematopoiesis, lead to chronic red cell destruction and anaemia. Conditions include red cell membrane disorders (such as hereditary spherocytosis), enzymopathies (for example G6PD deficiency), haemoglobinopathies and congenital dyserythropoietic anaemia. Disease severity in the mother varies from mild chronic anaemia to transfusion dependency, and in the fetus from being unaffected to hydrops fetalis. Diamond Blackfan syndrome may be also associated with physical anomalies in approximately 40%, and fetal loss has been reported to occur in up to 60% of cases.

Haemoglobinopathies

Haemoglobinopathy is defined as a group of inherited conditions in which there is abnormal production or structure of the haemoglobin molecule. Quantitative deficiency in globin chain synthesis causes impaired production and imbalance resulting in thalassaemia. Structural disorders cause qualitative abnormalities and result in variant haemoglobins, most commonly HbS. Comprehensive guidelines are available for the management of sickle cell disease and thalassamia in the UK, which include the management of pregnancy.

UK haemoglobinopathy screening service

The UK NHS Sickle and Thalassaemia Screening Program is a linked antenatal and neonatal program, launched in 2006. Antenatal screening aims to detect couples at risk of having a child with a major haemoglobinopathy before 12 weeks' gestation in order to offer prenatal diagnosis. Postnatally, all births in England are screened for haemoglobinopathy via the newborn heel prick sample with the primary aim of early detection of sickle cell disease (SCD).

Sickle cell disease

Sickle cell disease is an inherited autosomal recessive condition most commonly due to homozygous HbSS. Other compound heterozygous states such as HbSC, HbSβ-thalassamia or $HbSD_{punjab}$ also cause a sickling disorder with varying clinical severity. The clinical features of SCD are a result of chronic haemolytic anaemia and vasocclusion leading to painful crises, tissue ischaemia and infarction causing multiorgan damage.

Pregnancy associated with maternal SCD has an increased risk of maternal and fetal complications and should only be managed in specialist centres. Maternal sickle-related complications include worsening anaemia, increased painful crises, acute chest syndrome and infection. Maternal mortality is significantly increased and pregnancy-related complications occur more frequently, including hypertension, pre-eclampsia, thromboembolism and postpartum haemorrhage. Complications are generally fewer in women with HbSC disease, as compared to HbSS, except those effects of splenomegaly, such as thrombocytopenia, may occur and risk of venous thrombosis is higher. Risks to the fetus include increased miscarriage, intrauterine growth restriction, preterm delivery and stillbirth. The caesarean section rate is increased and perinatal mortality is reported at 4–6%.

Ideal management begins pre-conceptually with an assessment of pre-existing complications and optimisation of disease. Counselling about the risks of pregnancy should be undertaken and partner screening if appropriate. Pre-implantation genetic diagnosis can be offered to couples at risk of an affected child. Any teratogenic medication should be withheld, such as iron chelators, hydroxycarbamide and ACE inhibitors.

Antenatally, women should be reviewed at a minimum every 4 weeks until 24 weeks, every 2 weeks until 34 weeks and weekly thereafter. Blood pressure and urinalysis are required at each visit and an FBC monthly. In addition to routine standard scanning at 12 and 20 weeks, serial growth scans are offered every 4 weeks from 24 weeks.

Aspirin should be given between 12 and 36 weeks' gestation to reduce the risk of pre-eclampsia, and oral folic acid and penicillin prophylaxis should be continued. Hydroxycarbamide should be avoided if possible but risks vs benefits considered on an individualised basis. It is important that a plan is made for acute painful crisis occurring in pregnancy and fetal wellbeing is monitored during any admission with sickle crisis. VTE risk assessment is required and LMWH thromboprophylaxis offered from 28 weeks gestation, or earlier if there are additional risk factors for venous thrombosis, until 6 weeks postpartum. Screening for diabetes is recommended and a referral to the obstetric anaesthetic team should be planned for the third trimester. Prophylactic red cell transfusion is not routinely recommended but should be considered on an individualised basis, particularly in women with significant previous pregnancy complications and those previously on hydroxycarbamide.

Timing and mode of delivery are guided by obstetric indication, there is no evidence to support elective caesarean section for SCD alone. In view of the increased risk of complications in

the third trimester, with higher rates of stillbirth, placental insufficiency and abruption, many obstetricians would offer induction of labour at term. Hydration and oxygenation are advised during labour and continuous fetal heart monitoring recommended. Close monitoring in the postnatal period is recommended as the increased risk of painful crises continues.

Thalassaemia

Thalassaemia describes a group of disorders whereby there is an inherited quantitative defect in the production of otherwise normal globin chains. Although this most commonly affects the alpha (α) and beta (β) globin chains, thalassaemia of other chains such as gamma (ϒ) or delta (δ) are also described.

Classification is based on the type of globin chain production which is deficient. In complete absence of globin chain synthesis, the nomenclature α^0 or β^0 is used. When there is a reduced rate of synthesis, α^+ or β^+. The reduced rate of production of one of the globin chains results in an excess of the remaining normal chains; these precipitate in red cell precursors causing ineffective erythropoiesis, damaged red blood cells and chronic extravascular haemolysis. Further complexity occurs when a thalassaemic mutation is co-inherited with other thalassaemias or structural disorders.

Clinical classification is based on the severity of resulting anaemia and requirement for transfusion and a simplified version is shown in Table 53.1.

Thalassaemia carriage/trait

The mainstay of management for women with thalassaemia trait is determination of risk to the baby depending on paternal haemoglobin variants, and consideration of antenatal diagnosis. Maternal anaemia requires assessment and management of concomitant iron deficiency.

Table 53.1 Classification and haematological parameters of thalassaemia.

Syndrome	Genotypes	Phenotype
α Thalassaemia		
Normal	αα/αα	Normal
Silent carrier	−α/αα	Slight decrease MCV/MCH
Minor	−−/αα	Mild microcytic anaemia
	−α/−α	MCH <25 pg*
HbH disease	−−/−α	Moderate microcytic anaemia Hb 80–90 g/L
Hydrops fetalis with Hb Barts	−−/−−	Severe anaemia; usually fatal in utero
B Thalassaemia		
Normal	β/β	Normal
Minor (carrier or trait)	β/β^0	Mild microcytic anaemia
	β/β^+ **	HbA2 >3.5%*** Increased HbF
Non-transfusion-dependent (previously β thalassaemia intermedia)	β^+/β^+	Moderate microcytic anaemia
	β^0/β^+	HbA2 >3.5%***
	Coinheritance of other variants†	Increased HbF
Transfusion-dependent (previously β thalassaemia major)	β^0/β^0	Severe transfusion dependent microcytic anaemia
	β^0/β^+	Increased HbF
	HbE/β^0	

* MCH is used in the antenatal screening programme to identify women at risk of α^0 carriage and request partner screening.
** HbE is a β+ mutation.
*** Used in the antenatal screening programme to identify β thalassaemia and request partner screening.
† Other variants include Hb E (in combination with heterozygosity for a beta thalassaemia variant), hereditary persistence of fetal haemoglobin (HPFH; in combination with homozygous beta thalassaemia), alpha thalassaemia (in combination with homozygous β^+ thalassaemia), or triplicated or quadruplicated alpha genes (αα/ααα or αα/αααα; in combination with heterozygous beta thalassaemia). Dominantly acting beta thalassaemia variants can also occur.

All women known to the haematology service should be offered prenatal counselling about the management of pregnancy, including the offer of partner screening where appropriate. Specific treatment varies depending on the type of thalassamia.

Non transfusion dependent thalassamia

Pregnancy may exacerbate haemolysis in non transfusion dependent thalassamia (NTDT) such that some will require transfusion support. Hb should be monitored and transfusion considered for worsening maternal anaemia. In these cases, a pre-delivery Hb target of 100 g/L is advised. Maternal anaemia is a risk factor for fetal growth restriction (FGR) and regular growth scans are indicated from 24 weeks. Development of FGR is an indication of maternal transfusion support. In view of increased red cell turnover, folate supplementation (5 mg daily) is recommended.

Both NTDT and thalassaemia major (TM) (see below) are prothrombotic, especially if women have previously undergone splenectomy. Aspirin 75–100 mg daily should be offered to women who have undergone splenectomy or who have a platelet count $>600 \times 10^9$/L. If women have both, or if indicated by an individualised VTE risk assessment, low-molecular-weight heparin thromboprophylaxis should also be offered. All women should receive LMWH for 6 weeks postnatally.

Transfusion-dependent thalassaemia major

Individuals with TM require lifelong red cell transfusion support. This results in iron overload with subsequent cardiac, hepatic and metabolic bone disease. Endocrine dysfunction causes hypogonadotropic hypogonadism and subfertility, diabetes and hypothyroidism as well as small stature, which has implications for delivery. A full assessment of iron status and end-organ function is advised in advance of pregnancy.

Patients are routinely treated with iron chelation therapy from childhood. Preconceptually, chelation should be optimised as this can reduce overall iron burden and thus end-organ damage. Iron chelators are potentially teratogenic and deferasirox and deferiprone should be discontinued 3 months before conception. Desferrioxamine should be avoided in the first trimester due to lack of safety data but there is growing evidence that low-dose desferroxamine can be used safely in the second and third trimester. This should be considered in women with high risk of cardiac decompensation. Women with TM should receive chelation with intravenous desferrioxamine for the duration of labour to reduce the risk of free radical damage and cardiac dysrhythmia.

Women with TM require management in specialist centres with access to a multidisciplinary team including obstetricians, haematologists, cardiologist and diabetes specialist. Transfusion requirements tend to increase in pregnancy, and haemoglobin should be carefully monitored to maintain a baseline Hb of 100 g/L. Folic acid 5 mg daily is recommended and regular growth scans from 24 weeks to assess for FGR. The risk of haemolytic disease in the newborn needs to be considered in women with atypical red cell antibodies. Screening for diabetes is advised and thyroid function monitored.

Thrombocytopenia in pregnancy

Thrombocytopenia is common during pregnancy. Most cases are clinically mild with no intervention required and no adverse outcomes for mother or baby. Occasionally thrombocytopenia is more severe and cases can be associated with systemic disorders with implications for both mother and baby. Causes of thrombocytopenia in pregnancy are shown in Figure 53.1.

Gestational thrombocytopenia

Approximately 75% of cases are due to gestational thrombocytopenia, a benign condition most commonly occurring from the mid-second to third trimester. It typically resolves by 6 weeks postpartum but may recur in subsequent pregnancies. Thrombocytopenia is usually mild to moderate and the platelet count rarely falls $<70 \times 10^9$/L. There is no association with neonatal thrombocytopenia or bleeding.

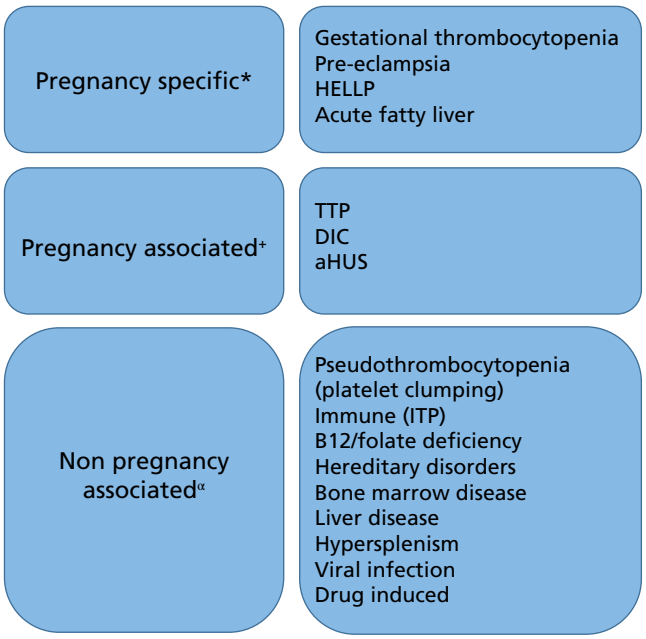

Figure 53.1 Causes of thrombocytopenia in pregnancy. *Occur only during pregnancy, +Occur in both pregnant and non-pregnant states but may be triggered by pregnancy or the postpartum period, αOccur in both pregnant and non-pregnant states. aHUS, atypical haemolytic uraemic syndrome; DIC, disseminated intravascular coagulation; HELLP, haemolysis elevated liver enzymes and low platelet syndrome; TTP, thrombotic thrombocytopenic purpura.

There is no diagnostic test for this condition; it remains a diagnosis of exclusion. Gestational thrombocytopenia (GT) is clinically most likely if the woman is well, with no previous personal or family history of thrombocytopenia or bleeding, a normal blood film and no other cause for thrombocytopenia evident. A normal platelet count outside of pregnancy is reassuring.

Woman can be counselled regarding the benign nature of the condition and the importance of presenting if they develop symptoms of bleeding. The platelet count should be monitored, particularly in the weeks leading up to delivery and the diagnosis reviewed if the platelet count falls below 70×10^9/L. A repeat FBC should be taken 6–8 weeks postnatally to ensure the platelet count has normalised.

Preeclampsia is the second most common cause of thrombocytopenia in pregnancy and, as with gestational thrombocytopenia, only occurs after 20 weeks gestation. Haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome is considered a variant of preeclampsia although in 20% hypertension is not a feature. Risk factors include multiple pregnancy and caucasian ethnicity. Epigastric pain is common, the haemolysis is microangiopathic with fragmented red cells, lactate dehydrogenase (LDH), aspartate transaminase and/or alanine transaminase are elevated and there is thrombocytopenia. The clinical and laboratory features are similar to thrombotic thrombocytopenia purpura (TTP) but the LDH:transaminase ratio is higher in TTP where hepatic impairment is unusual. Unlike TTP, HELLP improves with delivery of the baby, although in a minority of cases, symptoms progress in the early postpartum period before recovery occurs, and in 20% the is onset postpartum.

Immune thrombocytopenia

Immune thrombocytopenia (ITP) occurs in 2–3% of women who are thrombocytopenic in the third trimester and can be difficult to distinguish from GT. However, it is the commonest cause of thrombocytopenia if presenting in the first trimester. Approximately 2/3 of women have a pre-existing diagnosis whereas 1/3 are diagnosed during pregnancy.

Maternal considerations

There are few randomised trials to aid management of ITP in pregnancy with guidance based on consensus approach and expert opinion. Decisions regarding treatment are based on gestation, platelet count and urgency of platelet increment balanced against potential side effects of treatment. Approximately 1/3 of women require treatment during pregnancy.

Antenatally in the absence of bleeding, a platelet count of $20–30 \times 10^9$/L is thought to be safe. Consensus recommends a platelet count $>50 \times 10^9$/L for vaginal or caesarean birth and $>70 \times 10^9$/L for neuraxial anaesthesia. Consideration particularly should be given to the balance of risk between spinal anaesthesia in the context of thrombocytopenia vs the risks of general anaesthetic to facilitate operative delivery. A referral to the obstetric anaesthetic team should be made in advance of delivery.

First-line treatment is usually oral prednisolone, starting with 20 mg and titrated to the lowest dose possible to maintain a safe platelet count. Intravenous immunoglobulin (IVIG) can be used if there is a more urgent need to increase the platelet count but response is usually transient. A combination of steroids and IVIG can be used in refractory cases. Azathioprine and cyclosporin are safe in pregnancy but may take several weeks to achieve platelet response. There is growing experience with the use of Rituximab and this can be considered in severe refractory cases; however, a potential complication is neonatal immunosuppression and subsequent infection with monitoring required in the infant.

Data regarding the use of thrombopoetin receptor agonists (TPO-RA) in pregnancy are limited to small case series. Both romiplostim and eltrombopag are able to cross the placenta raising concern about potential harm to the fetus. However, there is positive experience with their use in the latter half of pregnancy for refractory cases. Splenectomy is rarely done and should ideally be performed laparoscopically in the second trimester.

Considerations in the fetus/neonate

Maternal autoantibodies are IgG and can cross the placenta with the potential to cause thrombocytopenia in the neonate. Approximately 10% of neonates born to mothers with ITP have platelets $<50 \times 10^9$/L and 5% have platelets $<20 \times 10^9$/L. There is no correlation between the neonatal and maternal platelet count; however, a previous history of neonatal thrombocytopenia is a strong predictor of recurrence. Severe maternal disease and a history of splenectomy are also considered risk factors for a low neonatal platelet count. As severe thrombocytopenia is unusual, very low platelets counts warrant exclusion of neonatal alloimmune thrombocytopenia (NAIT). In contrast to NAIT, the risk of intracranial haemorrhage in severe neonatal ITP is very low at <1%.

Mode of delivery is by obstetric indication. Interventions associated with an increased risk of neonatal haemorrhage such as fetal scalp blood sampling or electrodes, ventouse delivery and rotational forceps should be avoided. An umbilical cord platelet count should be taken at delivery and repeated at 3–5 days of age if low. Cranial USS should be performed if the platelet count is $<50 \times 10^9$/L. Symptomatic bleeding or a platelet count $<30 \times 10^9$/L are indications for treatment with IVIG.

Other autoimmune disorders

Autoimmune haemolytic anaemia

Haemolytic anaemia describes a shortened red cell lifespan. Increased erythropoiesis in the bone marrow is able to compensate when haemolysis is mild but as the rate of haemolysis exceeds capacity for compensation, anaemia occurs.

Autoimmune haemolytic anaemia (AIHA) is caused by the production of autoantibodies which bind to a red cell surface antigen resulting in premature removal; predominantly in the spleen, but also in the liver and occasionally intravascularly. AIHA can be classified as warm (predominantly IgG antibodies) or cold (mainly IgM or complement-fixing antibodies). It may be primary, or secondary to drugs, infection, autoimmune disease (typically systemic lupus erythematosus), neoplasia or other haematologic disorders.

Typical laboratory findings include anaemia, increased LDH and bilirubin, reticulocytosis and reduced haptoglobin; these are common to all causes of haemolytic anaemia. The distinguishing diagnostic test is the direct antiglobulin test (DAT or direct Coombs test) which is positive in immune-mediated haemolysis and negative in non-immune causes. The blood film shows polychromasia and spherocytosis with red cell agglutination seen in cold AIHA. Thrombocytopenia should prompt investigation for an alternative cause and red cell fragmentation requires urgent consideration of microangiopathic haemolytic anaemia (MAHA).

AIHA is seen only rarely during pregnancy, estimated to complicated 1:140,000 maternities. Conversely, anaemia is very common and therefore an index of suspicion is required in the case of a rapidly falling haemoglobin in the absence of another cause.

First-line treatment is with oral prednisolone, tapered to the minimum effective dose. Use of other immunosuppressive agents such as azathioprine, IVIG and rituximab has been described but the rarity of the condition means that there is little evidence upon which to base treatment decisions in steroid-refractory cases.

Supportive red cell transfusion may be required, however cross-matching red cells can be problematic. Autoantibodies coating the red cell surface have the potential to 'mask' underlying red cell alloantibodies causing difficulty in selecting suitable units for transfusion. Specialised laboratory testing may be required, which can delay the provision of red cells. Women with cold AIHA require transfusion through a blood warmer to avoid worsening red cell agglutination.

Haemolysis is a risk factor for thrombosis and consideration should be given to antenatal thromboprophylxaxis. All women require thromboprophylaxis during the highest risk time for thrombosis which is the first 6 weeks postpartum unless a contraindication exists. Oral folic acid 5 mg daily is prescribed to meet the increased folate requirements of red cell production.

IgG antibodies are able to cross the placenta and can cause antenatal or postpartum haemolysis in the fetus/neonate. Antenatally, women may require serial USS for growth and doppler USS of the fetal middle cerebral artery to screen for neonatal anaemia. Umbilical cord FBC, bilirubin and DAT should be taken at delivery and the neonate monitored for the development of anaemia and hyperbilirubinaemia. Although the DAT is often positive, severe fetal/neonatal haemolysis is rare. Management of neonatal AIHA is similar to that in haemolytic disease of the newborn (HDN).

Neutropenia

Neutropenia is defined as an absolute neutrophil count of $<1.5\times10^9$/L. It has many causes and most cases are mild. Neutropenia is described as severe if the neutrophil count is $<0.5\times10^9$/L and very severe if the count is $<0.2\times10^9$/L. There is ethnic variation in the normal neutrophil count with individuals of African and Caribbean descent typically having a lower count than their Caucasian counterparts.

Autoimmune neutropenia is a rare disorder caused by granulocyte-specific autoantibodies. Cases can be primary or secondary to other autoimmune conditions. Although testing for anti-neutrophil antibodies is possible, it is generally not helpful and does not guide management. Diagnosis is predominantly by exclusion of other causes.

Pregnant woman with neutropenia should be investigated in the same way as the non-pregnant patient including a careful history, review of medication and clinical examination. A peripheral blood film should be examined. Bone marrow examination can be performed safely in pregnancy if this is clinically indicated; however, this is rarely required unless a sinister cause is suspected.

The main maternal risk with severe neutropenia is sepsis which may be life-threatening, promote miscarriage or precipitate early labour. Close monitoring of maternal and fetal wellbeing is required. Infection should be treated promptly and consideration given to prophylactic antibiotics to cover invasive procedures, labour and delivery.

Outside of pregnancy, recombinant granulocyte colony-stimulating factor (GCSF) is used to treat patients with severe chronic neutropenia. Although there is limited data on the use of GCSF in pregnancy, adverse events have not been reported and the benefits are likely to outweigh the risks in a patient with repeated infections.

There is a risk of neonatal neutropenia and an umbilical cord sample should be taken for assessment of the full blood count. Neonatal infections are usually mild and serious morbidity from sepsis is rare; however, infants should be closely monitored for signs of infection.

Alloimmune disorders

Transplacental transfer of immunoglobulin G (IgG) begins early in the second trimester and fetal-maternal alloimmune disorders may arise from maternal sensitisation to fetal antigens, causing immune-mediated destruction of fetal cells. The most common alloimmune conditions are red cell alloimmunisation and NAIT with risk of fetal anaemia and thrombocytopenia, respectively.

Haemolytic disease of the fetus and newborn

Haemolytic disease of the fetus and newborn (HDFN) is most commonly due to RhD sensitisation, but anti-c and anti-Kell antibodies may be implicated as well as others at high titre (>1:32 dilution) such as anti E.

One in six RhD-negative women will sensitise after delivery of a term RhD-positive baby. Immune-mediated red cell destruction of a subsequent RhD-positive fetus gives rise to hyperbilirubinaemia. While the fetus is largely protected by placental passage, the newborn's immature liver is unable to conjugate bilirubin causing risk of kernicterus and long-term neurological sequelae. Furthermore, worsening fetal anaemia in utero leads to a compensatory increase in fetal erythropoiesis, initially in the bone marrow but subsequently dependant on extramedullary red cell production causing enlarged liver and spleen. As anaemia becomes more severe, cardiac output increases and without intervention, cardiac failure develops, leading to hydrops fetalis with widespread accumulation of extravascular fluid. In pregnancies at risk, detection of fetal anaemia by ultrasound scan of the fetus' middle cerebral artery guides management, with timely delivery or intrauterine red cell transfusion, to prevent this fatality.

Newborns need close observation. A cord sample should be taken for blood group, DAT, Hb and bilirubin. As bilirubin levels rise sharply after birth, repeat estimations may be required to determine the need for phototherapy. IVIG acts by blocking Fc clearance of red cells as well as inactivating circulating maternal immunoglobulin and, if anaemia is present, a single dose of IVIG 0.5–1.0 g/kg given shortly after birth reduces the need for exchange transfusion and ongoing top-up transfusions.

All women are screened for the development of atypical red cell antibodies in early pregnancy and at 28 week's gestation. For RhD-negative women, the fetal RhD type can be identified from a maternal blood sample, enabling counselling, a plan of management and if maternal antibody is not yet present, prevention of sensitisation with an injection of exogenous antiD, derived from pooled human plasma. This is offered at 28 weeks and after delivery to all RhD-negative women with a RhD-positive baby to prevent sensitisation and reduce risk to subsequent pregnancies.

Fetal neonatal alloimmune thrombocytopenia

Fetal neonatal alloimmune thrombocytopenia (FNAIT) occurs in approximately 1 in 1000 pregnancies and differs from red cell alloimmunisation in that the first pregnancy is often affected. Human platelet antigens (HPAs) are expressed on fetal platelets from 11 week's gestation and may cross the placenta and cause sensitisation in the mother whose immune system does not recognise the paternally-derived antigens. The anti-HPA antibodies may cause destruction of fetal platelets with ensuing thrombocytopenia and risk of bleeding. As there is direct targeting of fetal platelet antigens, thrombocytopenia can be severe, more so than caused by random binding of maternal autoantibody in ITP. Anti HPA 1a accounts for 80% of cases, anti HPA 5b 15% of cases, other anti-HPA antibodies are occasionally implicated.

Presentation is most commonly thrombocytopenia and bruising in the neonate but fetal intracranial haemorrhage (ICH) occurs in around 20% and may cause hydrocephalus, fetal demise or long-term neurological sequelae.

Diagnosis is made by serological testing for maternal anti-HPA antibody, paternal or neonatal genotype, and if necessary cross-match between mother's serum and father's platelets. The human leucocyte antigen (HLA)-DRB3*0101 in women is associated with clinically significant FNAIT and absence of this HLA has a negative predictive value of 99%.

The success of weekly IVIG therapy, usually from 16 week's gestation, has meant that invasive fetal blood sampling and platelet transfusion are no longer undertaken except before planned delivery when it may be beneficial to raise the platelet count to reduce risk of bleeding. Women at high risk, defined as a previous baby with an ICH or platelets under 20×10^9/L should receive IVIG 1 g/kg from 12 week's gestation with escalation of treatment at 20 weeks by doubling IVIG or adding steroids. Those at very high risk defined as ICH occurring before 28 weeks, should receive IVIG 2 g/kg weekly from 12 weeks with addition of steroids at 20 weeks.

Unlike for red cell antibodies, routine screening for anti-HPA antigens is not considered beneficial as it may lead to overdiagnosis by revealing transient antibodies. In addition, there is no way to prevent sensitisation, although research is underway on an anti-HPA-1a immunoglobulin to prevent women forming immune anti-HPA-1a. Anti-HPA 1a monoclonal antibodies are also in development which compete with maternal antibodies for binding to fetal HPA but unable to activate Fcg receptors and cause platelet destruction.

Physiological changes in coagulation status during pregnancy

To meet the needs of the developing fetus and placenta, uterine blood flow increases from 50 mL/min before pregnancy to over 1 L/min by term. The placenta has around 200 spiral arteries

which straighten and lose their vascular resistance so that almost 90% of the flow is directed to the placental intervillous spaces for transplacental exchange with the fetus. Not surprisingly, therefore, there is a high risk of obstetric haemorrhage during delivery when the placenta separates, particularly if contraction of the uterus is impaired.

To mitigate the risk of obstetric haemorrhage, there are physiological changes to the haemostatic system. Factors II, VII, VIII and X increase markedly with gestation and remain elevated in the early postpartum period. Factors V, IX and XI are unchanged. Factor XIII levels are lower in early pregnancy with a gradual increase in the second and third trimester. Fibrinogen concentration increases with a normal range of 4–6 g/L by the end of pregnancy. Von Willebrand factor (VWF) increases two to fourfold in the antenatal period which is thought to be due to oestrogen stimulation of endothelial cells to increase production of VWF. Levels peak during labour and delivery and begin to fall within the first few days after delivery, decreasing back to baseline in most women within 2–3 weeks.

The natural anticoagulant protein S decreases, protein C and antithrombin levels remain steady and there is increased resistance to activated protein C. Despite an increase in profibrinolytic proteins plasminogen and tissue-type plasminogen activator (tPA), overall fibrinolytic activity is reduced. This is due to an increase in inhibitors of fibrinolysis including thrombin activatable fibrinolytic inhibitor (TAFI) and plasminogen activator inhibitor 1 (PAI-1). During pregnancy, the antifibrinolytic protein PAI-2 is produced by placental villous cells, further adding to the anti-fibrinolytic effect. Fibrin degradation products, including the d-dimer, are increased, making this an unsuitable test to reliably exclude a diagnosis of VTE in the pregnant patient.

These physiological adaptations result in a shift in the balance of normal haemostasis to a prothrombotic, hypofibrinolytic state during pregnancy. Whilst this is necessary in order to maintain placental function and prevent haemorrhage at the time of delivery, these changes contribute to the increased thrombotic risk, which is well described in pregnant women.

Inherited bleeding disorders

Pregnant women with von Willebrand disease (VWD), carriers of haemophilia, or rare factor deficiencies are at increased risk of bleeding. VWF and Factor VIII levels increase from 6 to 8 weeks' gestation, reaching concentrations of three to fivefold baseline by term. Although this provides protection for delivery for women with haemophilia A carrier status and most women with VWD, they remain vulnerable in early pregnancy before levels peak, and in the puerperium when concentrations can fall abruptly. Desmopressin (DDAVP) increases release of stored FVIII and VWF, raising plasma levels threefold, and can be used to cover first-trimester procedures and the postpartum period. Tranexamic acid is useful to prevent excessive postpartum bleeding and is best given intravenously in established labour or by tablet after delivery.

Factor IX concentration does not change in pregnancy, and women with low factor IX (carriers of haemophilia B) may require recombinant factor concentrate for invasive procedures and delivery. Factor concentrations of 50% are generally considered safe for vaginal delivery and regional anaesthesia, although concentrations of 80% are preferred for caesarean section.

Inheritance risk to the baby needs to be considered and decisions made in early pregnancy and preferably pre-conceptually regarding prenatal diagnosis. Women who are carriers of severe haemophilia can be offered *in vitro* fertilisation with prenatal genetic diagnosis and embryo selection, or determination of fetal genotype in the early stages of pregnancy. Free fetal DNA can be extracted from a maternal blood sample from as early as 9 week's gestation for determination of fetal sex. If male, chorionic villous sampling for DNA analysis to determine haemophilia status can be undertaken between 10- and 12-weeks' gestation if results would influence the mother's decision whether to proceed with the pregnancy. Otherwise, amniocentesis at 34–36 weeks' gestation for genetic determination of haemophilia status allows informed decisions when planning delivery. Ventouse, rotational forceps, scalp electrodes and scalp sampling should be avoided if the male baby is affected with severe haemophilia.

UK guidelines on the management of inherited bleeding disorders in pregnancy have stratified risk for both mothers and babies according to disease type and severity. A summary is shown in Tables 53.2 and 53.3.

Obstetric haemorrhage

Maternal haemorrhage is a leading cause of maternal death. Haemorrhage commonly results from uterine atony as spiral arteries are then left open. Rarer causes include placental abruption, placenta praevia or accreta, and uterine rupture. Once blood loss exceeds 2000 mLs, dilutional coagulation deficits are common, exacerbating bleeding and ultimately leading to disseminated intravascular coagulation (DIC) and hypovolaemic shock when bleeding exceeds around 5000 mL. DIC can also be triggered by eclampsia, sepsis, retained products or amniotic fluid embolus and it is vital to consider the underlying cause of haemorrhage to determine the likelihood of early coagulopathy. All units should have a transfusion protocol for the management of massive obstetric haemorrhage such as that shown in Figure 53.2

Lessons learned from the UK and Ireland confidential enquiries into maternal deaths from haemorrhage include

- Optimisation of haemoglobin before delivery.
- Stimulating or augmenting uterine contractions in accordance with current guidance, paying particular attention to avoiding uterine tachysystole or hyperstimulation.

Table 53.2 Stratification of maternal bleeding risk in inherited bleeding disorders.

Risk level	Stratification of maternal bleeding risk	Suggested management of delivery (and note for postpartum care)
High risk	• Type 3 VWD • Severe (homozygous) rare coagulopathies • Severe platelet function disorders • Haemophilia carriers with a significant bleeding history	Adequate replacement therapy to be given after the onset of established labour. Avoid central neuraxial anaesthesia unless adequate replacement therapy has been confirmed. Maintain normal levels for at least 3 days postpartum or 5 days for caesarean section
Medium risk	• Type 2 VWD • Mildly reduced third-trimester maternal levels of factor VIII, IX or WWF activity • Factor XI deficiency with clinical bleeding phenotype	Adequate replacement therapy to be given after the onset of established labour. Avoid central neuraxial anaesthesia unless adequate replacement therapy has been confirmed. Maintain normal levels for at least 3 days postpartum or 5 days for caesarean section
Mild risk	• Mild platelet dysfunction disorders • Carriers for haemophilia with third-trimester factor VIII/IX levels ≥ 0.5 iu/mL • Type 1 VWD with corrected VWF activity >0.5 iu/mL	Consider tranexamic acid for delivery and postpartum.
Unlikely to be at risk	• Rare coagulopathies without bleeding phenotype (e.g. heterozygous factor V, VII, X, XI deficiency) • Factor XII deficiency (any severity)	No replacement therapy needed. Able to have central neuraxial anaesthesia.

Dysfibrinogenaemia tends to be associated with thrombosis but may cause bleeding.
VWD, von Willebrand disease; VWF, von Willebrand factor.
(*Source:* Pavord S *et al.* 2017/Royal College of Obstetricians and Gynaecologists.

Table 53.3 Stratification of Neonatal bleeding risk in Inherited Bleeding disorders.

Risk stratification of bleeding, according to possible or confirmed diagnosis of fetus		Suggested management of delivery
High Risk	Males with severe and moderate haemophilia A or B Type 3 vWD Severe (homozygous) rare coagulopathies or severe platelet disorders	Consider third-trimester amniocentesis. Discuss mode of delivery taking maternal and fetal factors into consideration, but avoid mid cavity forceps, ventouse, FBS, FSE, Cord sample for factor assay. Oral vitamin K, unless the result is known to be normal.
Medium Risk	• Males with mild haemophilia A or B • Type 2 vWD	• Consider third-trimester amniocentesis. • Avoid mid cavity forceps, ventouse, • Judicious use of FBS and FSE to facilitate vaginal delivery. • Cord sample for factor assay. • Oral vitamin K, unless the result is known to be normal.
Mild Risk	• Clinically moderate or severe type 1 vWD in family • Female fetuses who are obligate/possible carriers of severe haemophilia B • Mild platelet function disorders	• Consider avoiding ventouse and external cephalic version. • Judicious use of rotational forceps, FBS and FSE.
Unlikely Risk	• Clinically mild type 1 vWD in family • All other obligate/possible haemophilia carrier female fetuses • Heterozygous rare coagulopathies	No special precautions.

Source: Pavord S *et al.* 2017/Royal College of Obstetricians and Gynaecologists.

Major Obstetric Haemorrhage[A]

RESUSCITATE

Airways
Breathing
Circulation

Obstetric Measures

- ☐ Bimanual compression
- ☐ Ergometrine 500 mcg IV if no hypertension
- ☐ Syntocinon 10 iu IV & 40 iu infusion in 500 mls saline
- ☐ Check Placenta and Check for lacerations
- ☐ Carboprost IM 205 mcg Repeat at 15 minutes
- ☐ EAU
- ☐ Balloon tamponade
- ☐ Compression sutures
- ☐ Consider uterine artery ligation or internal iliaartery ligation
- ☐ Consider subtotal/total hysterectomy

Stand Down

Inform Laboratory and return unused Components.
Complete documentation
Attention to thrombosis

Call for help 2222[B]

State "Major Obstetric Haemorrhage" and Location
Call Consultant
Notify Cell Salvage Team

Blood Samples

- ☐ FBC and Crossmatch
- ☐ PT, APTT, Fibrinogen
- ☐ U+E, Ca2+
- ☐ POCT:ABG, HemoCue, TEG

Tranexamic acid 1g IV and
Start MHP1: Red Cells 4 units[C], FFP 4 units
Reconsider blood component use when TEG results available[E]

Reassess

Continuing haemorrhage
Repeat bloods
FBC, PT, APTT, Fibrinogen, U+E, Ca2+[D]
POCT: ABG, Haemocue, TEG

Order Products according to TEG and/or laboratory results Otherwise MHP2

- ☐ Red Cells 4 units
- ☐ FFP 4 Units
- ☐ Cryoprecipitate 2 pools
- ☐ Platelets 1 ATD Dose

Repeat Bloods:

FBC, PT, APTT, Fibrinogen, U+E, HemoCue, TEG
To inform further blood component requesting

PT – Prothrombin Time
APTT – Activated Partial Thromboplastin Time
FFP – Fresh Frozen Plasma

MHP - Massive Haemorrhage Pac
ATD – Adult Therapeutic Dose

*Based on local reference range using TEG6 analyser

[A] MOH Definitions

- ☐ >1000 ml and ongoing severe bleeding 150 ml/min
- ☐ Use of emergency GpO units
- ☐ Haemodynamic instability or sign of hypoperfusion
- ☐ Early signs of coagulopathy
- ☐ Other definitions may apply

[B] Call automatically transferred to Blood Transfusion Laboratory and Porters notified

[C] Emergency GpO Rhd-neg blood

- ☐ Emergency GpO RhD-positive blood if patient know to be RhD pos
- ☐ Group specific blood when ABP group and RhD knowns
- ☐ Cross-match blood
- ☐ AB or A plasma until Gp known

[D] Treatment targets:

Hb	80–100 g/l
R time	<7 min
K time	<2 min
α angle	>50°
MA	>60 μμ
Platelets	>75 × 10^9/l
PT ratio	<1.5
APTT ratio	<1.5
Fibrinogen	>2 g/l
Ca^{2+}	>1 mmol/l
pH	>7.35 (on ABG)

Monitor for hyperkalaemia
And maintain temp >36°C

[E] Response to TEG* results

R time	7–9 m	2 FFP
R time	>9 m	4 FFP AND 2 Cryo
α angle	<50°	2 Cryo
MA	52–60μμ	1 Plts
MA	<52μμ	2 Plts and 2 Cryo

POCT - Point of Care Testing
TEG – Thromboelastography

Figure 53.2 Example protocol for the management of obstetric haemorrhage (Source: Pavord and Maybury 2015/With permission of Elsevier.)

- Avoidance of delays to fluid resuscitation and blood transfusion,
- Being alert to warning signs and understanding that the normal physiological responses to haemorrhage may not be present early on, as young women compensate remarkably well. Although tachycardia commonly develops, there can be a paradoxical bradycardia and hypotension is always a very late sign, so continuing bleeding should be acted on without delay.
- Pre-empting coagulopathy, considering risk factors and providing blood components before coagulation indices deteriorate. This should include assessment and replacement of fibrinogen which when below 2 g/L or falling, is a predictor of severity of haemorrhage.
- Early recourse to hysterectomy if medical and surgical interventions are proving ineffective.

Patient blood management in obstetrics

Patient blood management (PBM) refers to the care of patients who may require a blood transfusion. Prevention of inappropriate or avoidable transfusions is particularly important in obstetrics due to the impact of any alloimmunisation on obtaining compatible blood in an emergency and to the fetus or newborn of haemolytic disease. Furthermore, pregnant women are significantly younger than the general population of transfused patients and potential unknown blood pathogens with long incubation times could impact their future health.

The three pillars of PBM; optimising erythropoiesis, minimising blood loss and managing anaemia, are addressed at different stages of pregnancy. The first requires adequate management of iron deficiency in the antenatal period to ensure normal Hb levels by term and adequate reserves in the event of haemorrhage at delivery. Iron deficiency also increases risk of postpartum haemorrhage, likely due to impaired uterine contractility.

Minimising blood loss in the intrapartum period involves prevention and prompt management of haemorrhage. Patients at risk should be identified and a delivery plan developed. Where unanticipated haemorrhage occurs, prompt multidisciplinary management is vital, considering the underlying cause of bleeding and likelihood of coagulopathy. Point of care viscoelastic testing (Thromboelastography [TEG] and rotational elastometry [ROTEM] can be helpful in the context of obstetric haemorrhage and whilst they do not replace laboratory tests, they can be performed within 10–15 min enabling rapid assessment of haemostasis facilitating prompt management. Local normal ranges and thresholds for intervention should be established, as shown in Figure 53.2.

Postpartum anaemia should be managed with iron supplements, and transfusion only given to a non-bleeding patient if Hb is under 70 g/L and there is cardiac compromise or risk of re-bleeding. If blood transfusion is necessary, information should be provided and consent obtained and only one unit should be given followed by reassessment.

Venous thromboembolism (VTE) in pregnancy

Pregnancy and the postnatal period are well-established risk factors for VTE, which remains the leading direct cause of maternal death in the UK. The combination of hypercoagulability, stasis of blood flow due to the gravid uterus and endothelial damage during delivery contribute to this risk. VTE can occur at any stage of pregnancy with the highest risk in the postnatal period where it is 25 times more common than in the non-pregnant population. VTE can present as an isolated deep vein thrombosis (DVT), or embolisation resulting in pulmonary embolism (PE). All women in the UK undergo individualised VTE risk assessment in early pregnancy which is repeated as clinical status changes, in accordance with guidelines from the Royal College of Obstetricians and Gynaecologists. Thromboprophylaxis with low molecular weight heparin (LMWH) is offered to those at the highest risk.

Diagnosis

Other than an increased propensity for the left leg (90% of DVT during pregnancy), the presentation of DVT and PE is the same as the non-pregnant population. Clinical assessment in both cases is unreliable. The higher index of suspicion for VTE during pregnancy and the overlap of symptoms with other pregnancy morbidity reduces the accuracy of clinical diagnosis to approximately 8% for DVT and less than 5% for PE. To add to the diagnostic difficulty, the Wells score is not validated in pregnancy and the d-dimer physiologically increases throughout normal pregnancy making this an unreliable marker for exclusion of VTE. Objective testing is therefore required in women with suspected thrombosis.

Compression duplex ultrasound scan (USS) is the primary diagnostic test for DVT. If the USS is negative and a high level of clinical suspicion exists, anticoagulant treatment should be discontinued but the USS is repeated on days 3 and 7. For the diagnosis of iliac vein thrombosis, USS of the iliac vein, MRI venography or conventional contrast venography may be considered.

Women with suspected PE who have symptoms and signs of DVT should have initial USS performed; if DVT is confirmed, no further investigation for PE is required. All other women require an initial CXR followed by ventilation/perfusion (VQ) scan or computerised tomography pulmonary angiogram (CTPA). Choice of imaging will depend on local availability and patient preference. CTPA is the imaging technique of choice if the CXR is abnormal. Expert consensus recommends VQ scanning where possible due to the high negative predictive value, the lower radiation dose to the maternal breast and the reduced likelihood of co-existing lung pathology in the young pregnant

population. Women should be advised that there is a slightly increased risk of childhood cancer with VQ scanning but the absolute risk is very small.

Management

Baseline FBC, renal and liver function are required prior to treatment. Therapeutic anticoagulation with LMWH is recommended at suspicion of VTE; unlike vitamin K antagonists such as warfarin, LMWH does not cross the placenta and has a good safety record for use in pregnancy. During pregnancy, changes in volume of distribution and renal glomerular filtration rate result in alterations in the pharmacokinetics of LMWH and historically, guidelines have recommended a twice-daily dosing regimen for enoxaparin and dalteparin. More recent evidence supports safety of a once-daily dosing regimen; however, there remains uncertainty regarding the optimum dosing for pregnant women.

LMWH should be continued throughout pregnancy. It is not established whether it is safe to recommend a dose reduction after the initial period of anticoagulation although this practice is employed successfully in some centres. Monitoring of anti-Xa levels is not routinely required unless there are concerns of renal impairment, extremes of body weight or a known diagnosis of antithrombin deficiency. Monitoring of platelet count is not required as heparin-induced thrombocytopenia (HIT) is extremely rare in pregnancy.

Massive life-threatening PE

Massive life-threatening PE is an emergency. Women present with haemodynamic compromise, hypoxaemia and evidence of right ventricular dysfunction. Cardiorespiratory arrest may ensue. Immediate assessment by a multidisciplinary team of experienced physicians, obstetricians, haematologists and interventional radiologists is required with assessment on an individual basis whether a woman receives intravenous unfractionated heparin (IV UFH), thrombolytic therapy or thoracotomy and surgical embolectomy

IV UFH is the preferred, initial treatment in massive PE due to its rapid effect and extensive experience of use. It can also be adjusted easily if thrombolytic therapy is required. Urgent portable ECHO or CTPA is advised within an hour of presentation however in extreme circumstances immediate thrombolysis may be indicated.

Delivery

Consideration should be given to planned delivery to avoid the risk of labour with full-dose anticoagulation. Therapeutic LMWH should be discontinued 24 hours prior to planned operative delivery, or 12 hours for prophylactic LMWH. In the event of spontaneous labour, women should be advised to omit LMWH and attend for review. For VTE occurring very close to term, IV UFH is an option due to the short duration of activity. Protamine sulphate to reverse heparin should be available for use if required. Unlike UFH, protamine only achieves partial reversal of LMWH and tranexamic acid may be a helpful adjunct for women in labour on therapeutic LMWH.

Regional anaesthetic techniques are not recommended until at least 24 hours after the last dose of therapeutic LMWH. LMWH should not be given for 4 hours after the use of spinal anaesthesia or removal of epidural catheter, and the epidural catheter should not be removed within 12 hours of the most recent injection. Referral to the obstetric anaesthetic team is advised.

Postnatal management

A prophylactic dose of LMWH can be given 4 hours after delivery. Although some guidance recommends that therapeutic LMWH can be recommenced as early as 8–12 hours after delivery, many centres build up more gradually over 48 hours, particularly in the event of caesarean section, to avoid the risk of wound haematoma. Therapeutic anticoagulation should be given for a minimum of 6 weeks postnatally and no less than 3 months of total treatment. LMWH and warfarin can both be used safely in breastfeeding women. Although there is emerging evidence for the use of direct oral anticoagulants (DOACs) in breast-feeding women, these are currently not recommended for routine use pending more data.

Bone marrow disorders

Myeloproliferative neoplasms

Pregnancy in women with myeloproliferative neoplasms (MPN) may be complicated by thrombosis or haemorrhage and around 30% risk of first-trimester fetal loss, higher with polycythaemia vera. Management should start before conception with counselling about risks, optimisation of the condition and general health, and withdrawal of potentially teratogenic medications such as hydroxycarbamide and anagrelide. Haematocrit <45% and blood counts should be controlled, with venesection, interferon and platelet- or leukapheresis as necessary. Aspirin helps prevent fetal loss, and heparin thromboprophylaxis may be necessary if additional risk factors for venous thrombosis are present. An algorithm for management of MPN in pregnancy is shown in Figure 53.3.

Haematological malignancy

Leukaemia or lymphoma diagnosed during pregnancy poses difficult dilemmas for the woman, her family and the multidisciplinary team. Timing of chemotherapy depends on the health

of the mother, the natural course of the condition, teratogenicity of its treatment and the gestation and progress of the pregnancy. In general, chemotherapy and radiotherapy in the first trimester are associated with significant risks of congenital malformations and miscarriage which lessens with advancing pregnancy.

For women with previous haematological malignancy, late effects of chemotherapy such as anthracycline-induced cardiomyopathy and bleomycin-induced pulmonary fibrosis should be considered and appropriate screening tests performed.

Aplastic anaemia and paroxysmal nocturnal haemoglobinuria

Pregnancy outcomes in women with aplastic anaemia are generally good, depending on the degree of bone marrow suppression. Regular monitoring is required to guide appropriate support with red cell and platelet transfusions, and prompt antibiotics for infections. Most cases of acquired aplastic anaemia are autoimmune and if cytopenia becomes severe, anti-thymocyte globulin (ATG), methylprednisolone and/or Cyclosporin can be given.

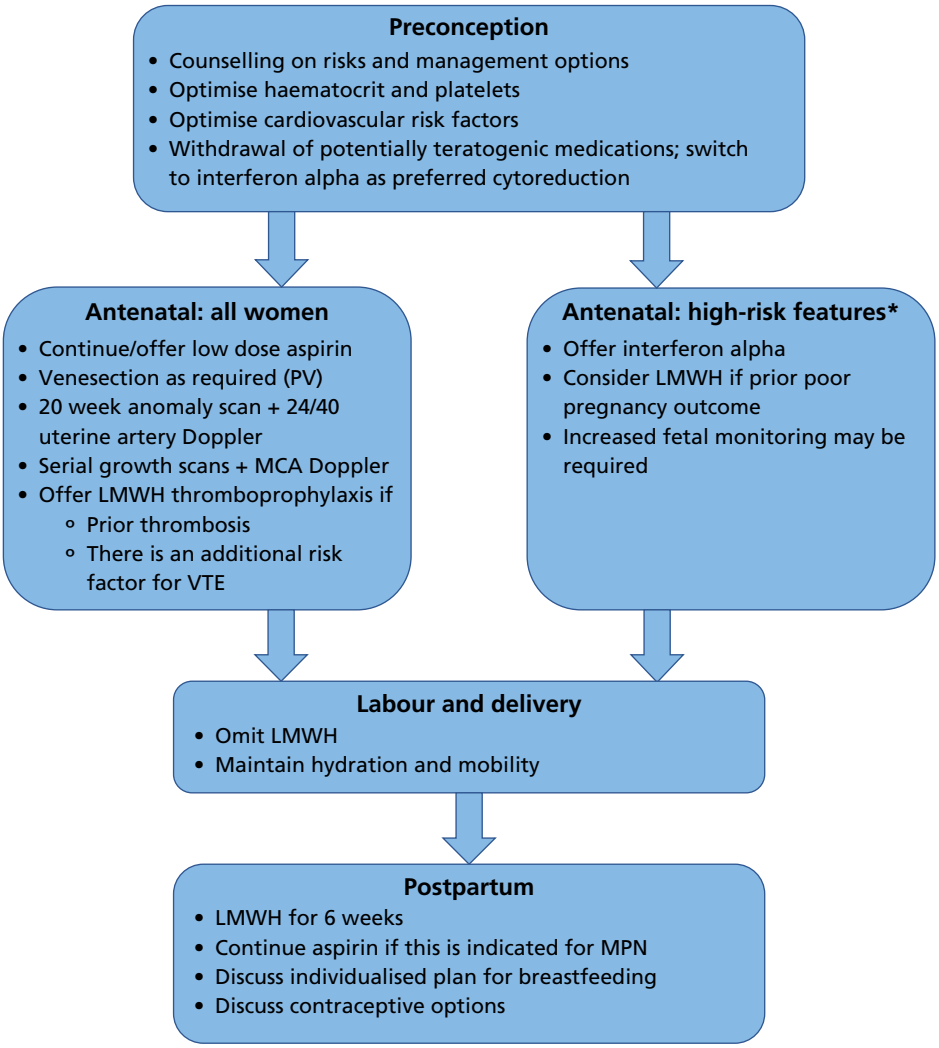

Figure 53.3 Management of MPN in pregnancy. *High-risk pregnancy criteria may suggest an increased risk of fetal or maternal complications. Previous venous or arterial thrombosis, previous haemorrhage attributed to MPN. Previous pregnancy complication towards which MPN may have contributed: Three pregnancy losses <12 weeks or one or more pregnancy loss >12 weeks. Intrauterine growth restriction, preeclampsia or other evidence of placental dysfunction. Intrauterine death or stillbirth with no other cause identified. Antepartum or major postpartum haemorrhage requiring red cell transfusion. Platelet count rising to >1500 × 10^{-9}/L before or during pregnancy$^{\Delta}$. Diabetes or hypertension requiring treatment$^{\Delta}$. $^{\Delta}$ These criteria would be an indication for cytoreductive treatment but not LMWH.

Associated paroxysmal nocturnal haemoglobinuria (PNH) increases risk of maternal and fetal morbidity and mortality and requires consideration of thromboprophylaxis and transfusion support for intravascular haemolysis. Risks and benefits of complement inhibitor treatment should be discussed but it is likely necessary to continue pre-pregnancy management to maintain stability for the pregnancy.

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CHAPTER 54

Neonatal haematology

54

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Developmental haemopoiesis

In humans, haemopoiesis begins in the yolk sac in the third week of gestation and moves sequentially to the aorto–gonado–mesonephros (AGM) by 5 weeks gestation, the liver by 6–8 weeks and the bone marrow around the 11th week of gestation. The AGM involutes early in the first trimester and the liver is the principal site of haemopoiesis until the end of the third trimester. Fetal haemopoietic stem cells (HSCs) have different molecular and functional characteristics compared to adult HSCs. These differences ensure that fetal HSCs meet the specific needs of the fetus for different types of mature blood cells. The predominant lineage during fetal life is erythropoiesis, but platelets and all types of leucocyte found in adult blood are also seen from as early as 4–5 weeks gestation.

There are a number of differences between erythropoiesis in neonates and adults: red cell morphology is distinctive with large numbers of crenated red cells, particularly in preterm neonates (Figure 54.1); red cell lifespan is reduced (35–50 days in preterm infants, 60–70 days in term infants); susceptibility to oxidant-induced injury is increased because of differences in the glycolytic and pentose phosphate pathways; the erythropoietin response to anaemia is blunted and specific embryonic and fetal globin chains are synthesised (Table 54.1). The first globin chain produced is ε-globin, followed by α- and γ-globin chains. Haemoglobin F (HbF, $\alpha_2\gamma_2$) is produced from 4–5 weeks' gestation and is the predominant haemoglobin until after birth. Adult haemoglobin (HbA, $\alpha_2\beta_2$) is produced from 6–8 weeks' gestation, but remains at low levels until after birth. In term babies, the average HbF at birth is 70–80%, the HbA is 25–30%, there are small amounts of HbA_2 and sometimes a trace of Hb Barts (γ_4).

Immediately after birth, rates of haemoglobin synthesis and red cell production fall in response to the sudden increase in tissue oxygenation at birth. In term babies, the haemoglobin reaches a mean of 130–140 g/L at the age of 4 weeks and 95–110 g/L at 7–9 weeks of age. Studies of well-preterm infants show a steeper fall in haemoglobin, reaching a mean of 65–90 g/L at 4–8 weeks of age. The reticulocyte count falls soon after birth as erythropoiesis is suppressed and increases back to normal values at 6–8 weeks of age. The blood volume at birth varies with gestational age and the timing of clamping of the cord. In term infants, the average blood volume is 80 mL/kg and in preterm infants 106 mL/kg (range 85–143 mL/kg). Term and preterm babies have adequate stores of iron, folic acid and vitamin B_{12} at birth. However, stores of both iron and folic acid are lower in preterm infants and are depleted more quickly, leading to deficiency after 2–4 months if the recommended daily intakes are not maintained.

Neonatal anaemia

Definition and pathophysiology

Any neonate with a haemoglobin of less than 130 g/L at birth should be considered anaemic and may require investigation (Figure 54.2). However, it is important to be aware that the haemoglobin concentration is affected by the site of sampling (it is up to 40 g/L lower in venous samples than in heel-prick samples in the first few days of life) and the timing of the clamping of the

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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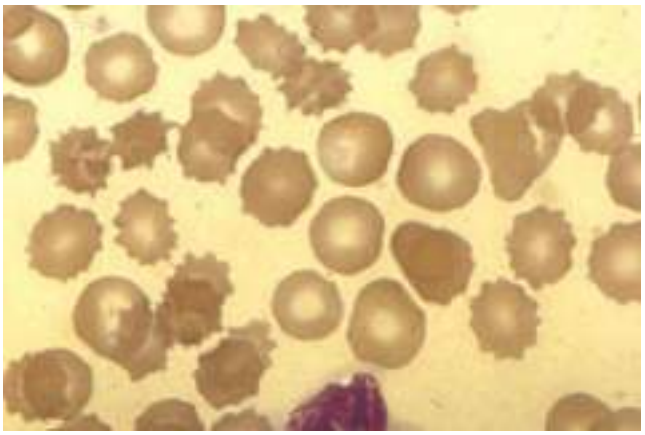


Figure 54.1 Typical erythrocyte morphology in a preterm neonate. Crenated red cells are a normal feature of the blood film of preterm neonates during the first few weeks of life. This film is from a neonate born at 26 weeks' gestation and shows the number of crenated cells present in neonates under 28 weeks' gestation. The number of these cells is inversely proportional to gestational age at birth.

cord (around 30 g/L higher after late clamping). The clinical significance of neonatal anaemia depends on whether the baby is able to maintain adequate tissue oxygenation. This in turn depends on the position of the haemoglobin–oxygen dissociation curve, which is principally determined by the concentrations of HbF and 2,3-diphosphoglycerate (2,3-DPG); a high HbF and low 2,3-DPG both shift the curve to the left, i.e. the affinity of haemoglobin for oxygen is increased and less oxygen is released to the tissues. This is the situation just after birth and may be more of a problem for very preterm babies as their HbF levels are greater than 90%. Over the first few months of life, 2,3-DPG levels rise and HbF levels fall so that the haemoglobin–oxygen dissociation curve gradually shifts to the right, the oxygen affinity of haemoglobin falls and oxygen delivery to the tissues increases, ameliorating the effects of the falling haemoglobin.

Table 54.1 Composition of haemoglobins in the human embryo, fetus and neonate.

	Globin chains		
Haemoglobin	**α-Globin gene cluster***	**β-Globin gene cluster***	**Gestation**
Embryonic			
Hb Gower 1	ξ_2	ε_2	From 3–4 weeks
Hb Gower 2	α_2	ε_2	
Hb Portland	ξ_2	γ_2	From 4 weeks
Fetal			
HbF	α_2	γ_2	From 4 weeks
Adult			
HbA	α_2	β_2	From 6–8 weeks
HbA_2	α_2	δ_2	From 30 weeks

* The α-globin gene cluster is situated on chromosome 16 and the β-globin gene cluster on chromosome 11. Note that fetuses and neonates with α-thalassaemia major, who are unable to synthesise α-globin chains, will have Hb Portland as well as Hb Barts (β_4), detectable by haemoglobin electrophoresis or HPLC.

Causes of neonatal anaemia

Anaemia may be caused by reduced red cell production, increased red cell destruction (haemolysis) or blood loss (Table 54.2).

Neonatal anaemia due to reduced red cell production

The main diagnostic clues to reduced red cell production are the combination of a low reticulocyte count ($<20\times10^9$/L) with a negative direct antiglobulin test (Coombs test). The most common causes are congenital parvovirus B19 infection and genetic red cell aplasias, particularly Diamond–Blackfan anaemia (DBA).

Parvovirus B19 and fetal/neonatal anaemia

Maternal infection with parvovirus B19 can cause severe fetal anaemia and in 9% of cases leads to intrauterine death. The baby has marked reticulocytopenia (often $<10\times10^9$/L) and thrombocytopenia may also occur. The diagnosis is made by maternal serology and demonstration of B19 DNA in the fetus or neonate by dot-blot hybridisation or polymerase chain reaction (PCR) of peripheral blood (bone marrow aspiration for morphology and parvovirus B19 PCR may be necessary in difficult cases). Severe cases require intrauterine transfusion, but have a good long-term outcome if they survive to delivery.

Genetic red cell aplasia

Apart from DBA, the genetic causes of congenital red cell aplasia are extremely rare. They include congenital dyserythropoietic anaemia (CDA) and Pearson syndrome; haematological evidence of the other inherited bone marrow failure syndromes, such as Fanconi anaemia, are almost never present at birth. These genetic causes of congenital red cell aplasia can normally be distinguished from each other through a combination of their distinctive bone marrow morphology, family history and the presence, or not, of additional congenital anomalies.

DBA

The incidence of DBA is estimated at five to seven cases per million live births. DBA has a clear family history in 20% of cases (autosomal dominant or recessive) and appears to be sporadic

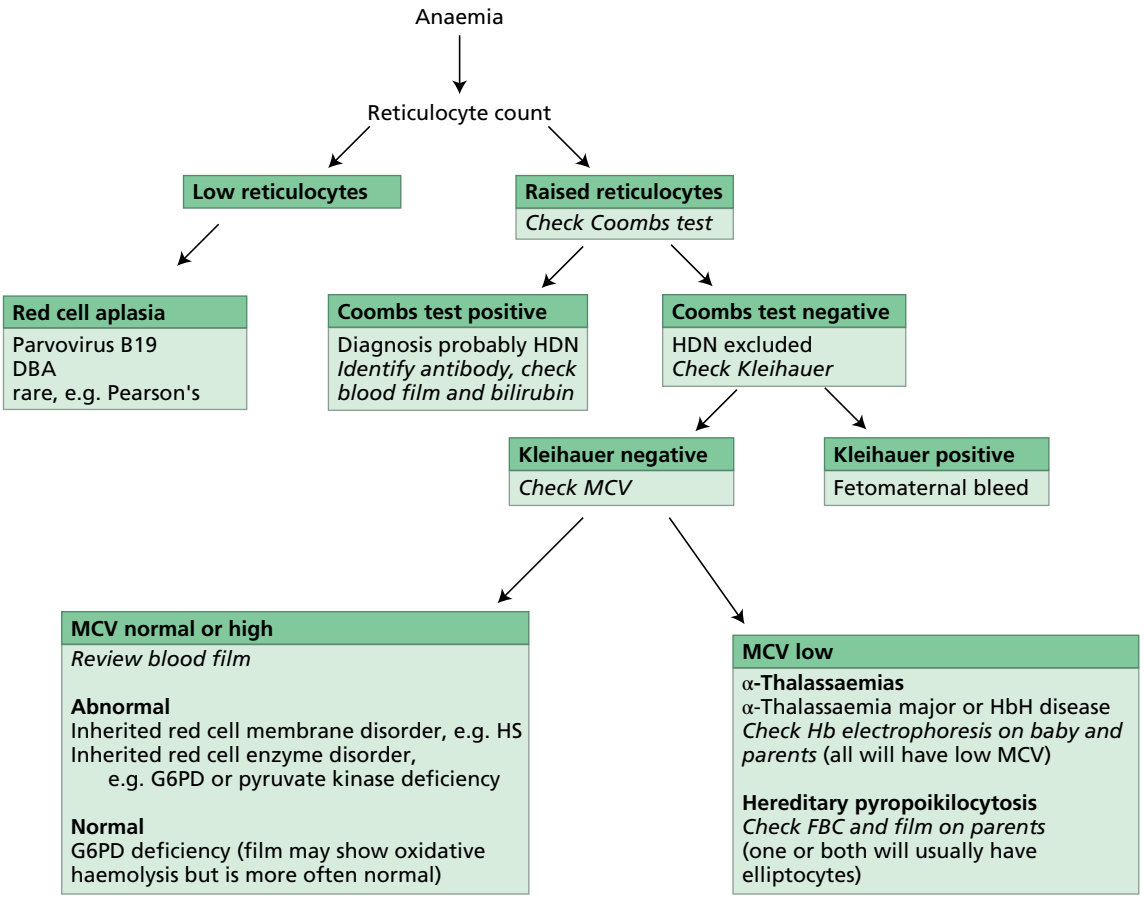


Figure 54.2 A diagnostic algorithm for neonatal anaemia. The most useful screening tests for investigating unexplained neonatal anaemia are the reticulocyte count, the Coombs' test and the mean corpuscular volume (MCV) of the red cells. DBA, Diamond–Blackfan anaemia; G6PD, glucose-6-phosphate dehydrogenase; HDN, haemolytic disease of the newborn; HS, hereditary spherocytosis.

in the remaining 80%. Presentation in the neonatal period or first few months of life with increasing anaemia from birth is typical, but more severe cases manifest as second-trimester anaemia or hydrops fetalis. Around 40% of infants have associated congenital anomalies, particularly craniofacial dysmorphism, neck anomalies and thumb malformations similar to those seen in Fanconi anaemia. In neonates, the blood film shows normochromic red cells with an absence of polychromasia and nucleated red cells despite severe anaemia (Figure 54.3). Reticulocytopenia is usually severe, but automated reticulocyte counts of $20–30 \times 10^9$/L are sometimes seen. Erythroid precursors are very low or absent in the bone marrow aspirate. These features are diagnostic of DBA if parvovirus infection is excluded. Raised red cell levels of adenosine deaminase (ADA) in the patient and/or parents may be useful for confirming the diagnosis, although normal red cell ADA levels do not exclude DBA. Recent studies indicate that most cases of DBA are due to defects in the synthesis and function of structural ribosomal proteins (RP). At present mutations in 20 *RP* genes have been identified and account for about 80% of DBA cases, *RPS19* mutations being the commonest (25% of all DBA). Additionally, DBA- like phenotype has been identified in patients with recessive inheritance with mutations in the *EPO* gene, and X-linked inheritance with mutations in *GATA1* and *TSR2* genes. In approximately 20% of patients, the molecular basis for the disease has proved elusive, even after extensive sequencing using targeted next generation sequencing and screening for large deletions. It is expected that further work with whole genome sequencing will help find some of these.

In the neonatal period, the only treatment of DBA is red cell transfusion, although steroids are used in older infants and children.

Pearson syndrome

This rare condition nearly always presents within a few days or weeks of birth. Pearson syndrome is caused by mutations or deletions in mitochondrial DNA and presents with normochromic anaemia, neutropenia, thrombocytopenia and failure to thrive. Most affected neonates have low birth weight, and metabolic acidosis, exocrine pancreatic deficiency and

Table 54.2 Common of neonatal anaemia.

Reduced red cell production
- Diamond–Blackfan anaemia
- Congenital viral infections, e.g. parvovirus, cytomegalovirus
- Congenital dyserythropoietic anaemia
- Pearson syndrome

Increased red cell destruction (haemolysis)
- Alloimmune: haemolytic disease of the newborn (Rh, ABO, Kell, other)
- Red cell membrane disorders, e.g. hereditary spherocytosis
- Red cell enzyme deficiencies, e.g. pyruvate kinase deficiency
- Some haemoglobinopathies, e.g. α-thalassaemia major, HbH disease

Blood loss
- Occult haemorrhage before or around birth, e.g. twin–twin, fetomaternal
- Internal haemorrhage, e.g. intracranial, cephalhaematoma
- Iatrogenic, due to frequent blood sampling

Anaemia of prematurity
- Impaired red cell production plus reduced red cell lifespan

abnormal liver and renal function are common. The most useful haematological clue to the diagnosis is the highly characteristic vacuolation of early erythroid cells on the marrow aspirate (Figure 54.4). Interestingly, large mitochondrial gene deletions have also been reported in a small subgroup of DBA patients. When these cases were re-evaluated, they were found to be

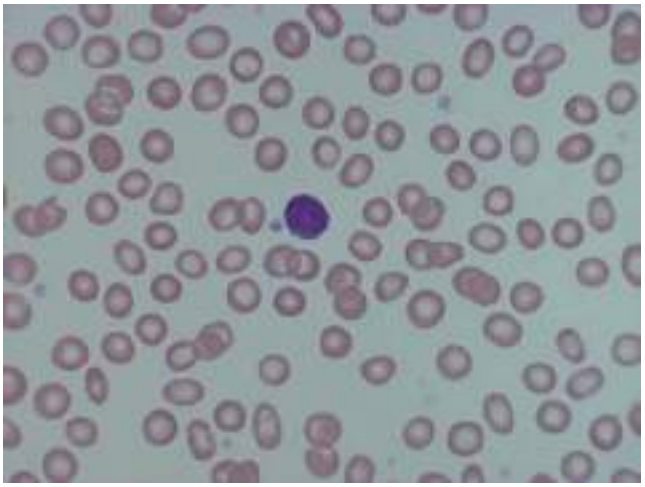


Figure 54.3 Blood film from a neonate with Diamond–Blackfan anaemia. This baby presented with fetal anaemia at 20 weeks' gestation and received intrauterine transfusion. At birth, the baby had normochromic anaemia and the blood film showed a complete absence of polychromasia and nucleated red cells, despite a haemoglobin of 70 g/L.

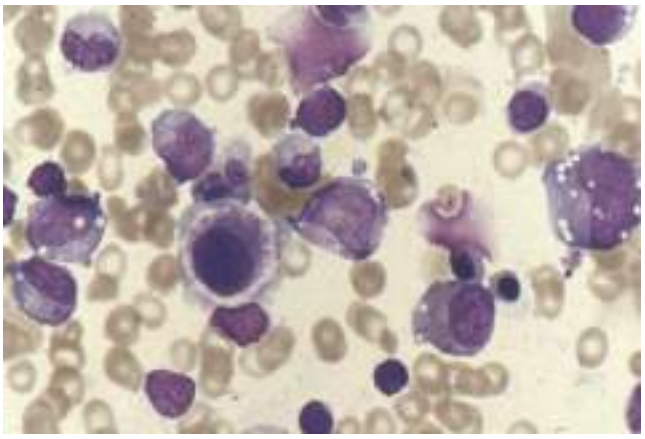


Figure 54.4 Pearson syndrome. Bone marrow aspirate from a neonate with Pearson syndrome showing typical vacuolation of erythroblasts and a dysplastic megakaryocyte.

cases of Pearson syndrome which had been misdiagnosed as DBA. Unfortunately, the prognosis for children with Pearson syndrome is very poor, with few surviving beyond the second year of life.

CDA

The clinical presentation of CDA is extremely variable. While the most severe forms may present as hydrops fetalis in association with fetal anaemia, milder forms of the disease may not present until adulthood. CDA is conventionally classified into three main types (I, II and III) with additional variant types of CDA. Most case reports of CDA presenting in the neonatal period are Type I or CDA variants. CDA type I is caused by mutations in the *CDAN-1* gene or, occasionally, in the *C15ORF41* gene. Type II CDA is due to mutations in the *SEC23B* gene and CDA type III, which is very rare, is due to mutations in the *KIF23* gene, while some variant forms of CDA are due to mutations in *LPIN2* or in the transcription factor genes *GATA1* or *KLF1*. Due to the widespread availability of newer DNA sequencing technologies, several other genes such as *PARP4, VPS4A* and *PRDX2* have recently been identified in a few pedigrees with CDA-like anaemias but the pathogenicity of these variants is yet to be conclusively validated.

Neonatal anaemia due to increased red cell destruction (haemolysis)

The main diagnostic clues suggesting a haemolytic anaemia are increased numbers of reticulocytes and/or circulating nucleated red cells (Figure 54.5), unconjugated hyperbilirubinaemia, a positive Coombs' test (if immune) and characteristic changes in the morphology of the red cells on a blood film (e.g. hereditary spherocytosis; Figure 54.5). The main cause of immune haemolytic anaemia is haemolytic disease of the newborn. The main

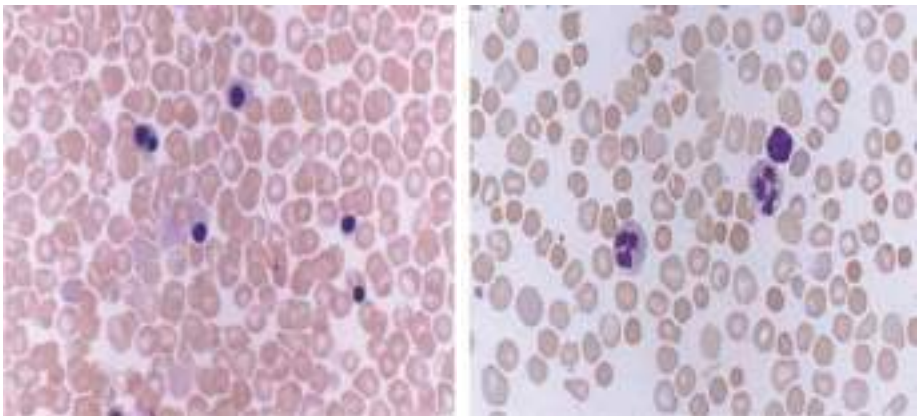

(a) (b)

Figure 54.5 Haemolytic disease of the newborn. (a) Blood film from a baby with rhesus haemolytic disease of the newborn due to anti-D showing polychromasia and large numbers of nucleated red cells, but relatively few spherocytes. (b) Blood film from a neonate with ABO haemolytic disease of the newborn due to anti-A, showing very large numbers of spherocytes, polychromasia and no nucleated red cells.

causes of non-immune neonatal haemolysis are red cell membrane disorders, red cell enzymopathies and, occasionally, haemoglobinopathies (Table 54.2).

Immune haemolysis, including haemolytic disease of the newborn

The principal alloantibodies causing haemolytic disease of the newborn are those against Rh antigens (anti-D, anti-c and anti-E), anti-Kell, anti-Kidd (J^k), anti-Duffy (F^y) and antibodies of the MNS blood group system, including anti-U. Anti-D is the most frequent alloantibody causing significant haemolytic anaemia, affecting 1 in 1200 pregnancies. Anti-Kell antibodies are less common but can cause severe fetal and neonatal anaemia as they inhibit erythropoiesis as well as causing haemolysis. Most babies with haemolytic disease of the newborn present with jaundice and/or anaemia; in severe cases, there is hepatosplenomegaly and/or skin deposits due to extramedullary haemopoiesis. ABO haemolytic disease occurs only in offspring of women of blood group O and is confined to the 1% of such women who have high-titre IgG antibodies. Haemolysis due to anti-A is more common (1 in 150 births) than that due to anti-B. In contrast with anti-Rh antibodies, both anti-A and anti-B usually cause hyperbilirubinaemia without significant anaemia; however, hydrops has occasionally been described. The blood film in ABO haemolytic disease characteristically shows very large numbers of spherocytes with little or no increase in nucleated red cells (see Figure 54.5a); this contrasts with rhesus haemolytic disease of the newborn, in which there are few spherocytes and large numbers of circulating nucleated red cells (see Figure 54.5b). Non-invasive prenatal testing of maternal blood samples for cell-free fetal DNA for relevant fetal antigens is currently in routine use in some countries to assess fetal genotype in women with alloantibodies in order to help with management of pregnancy.

Management of haemolytic disease of the newborn: All neonates at risk should have cord blood taken for measurement of haemoglobin, bilirubin and a Coombs' test and should remain in hospital, or under close outpatient supervision, until hyperbilirubinaemia and/or anaemia have been properly managed. Rhesus-alloimmunised infants with haemolysis should receive phototherapy from birth, as the bilirubin can rise steeply; this prevents the need for exchange transfusion in some infants. In haemolytic disease due to anti-Kell, anaemia is usually more prominent than jaundice and minimal phototherapy may be necessary despite severe anaemia. ABO haemolytic disease of the newborn usually just requires phototherapy, as anaemia is uncommon.

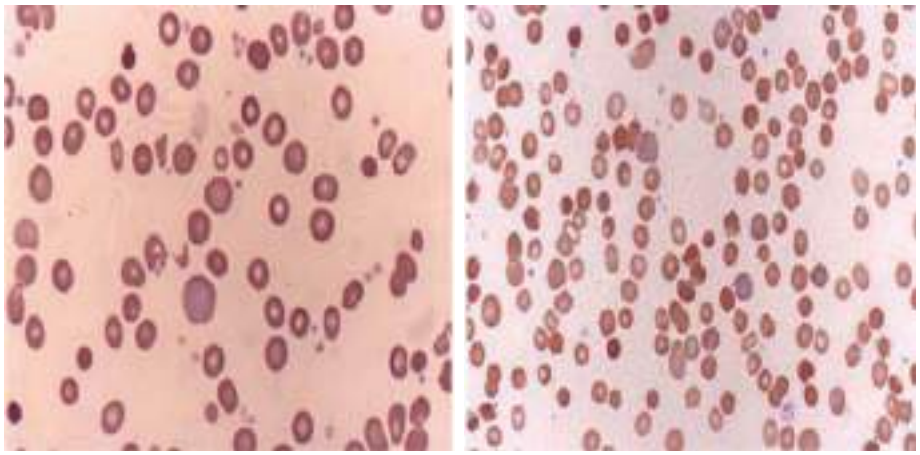
The indications for exchange transfusion in haemolytic disease of the newborn are:

- severe anaemia (haemoglobin <100 g/L at birth) and/or
- severe or rapidly increasing hyperbilirubinaemia.

The British Society for Haematology (BSH) has published useful guidelines for neonatal exchange transfusion. Intravenous immunoglobulin can be used to reduce the need for exchange transfusion, but the value of this approach remains to be proven in clinical trials. Neonates with haemolytic disease due to anti-Rh antibodies may develop 'late' anaemia at a few weeks of age, requiring 'top-up' transfusion; irradiated blood must be used for infants previously receiving intrauterine transfusion to prevent the risk of transfusion-associated graft-versus-host disease. Recombinant erythropoietin sometimes prevents the need for top-up transfusion for late anaemia but is not effective when haemolysis is brisk and there are currently no data from randomised trials to support its routine use in this setting. Folic acid (500 µg/kg daily) should be given to all babies with ongoing haemolysis until they reach 3 months of age.

Neonatal haemolytic anaemia due to red cell membrane disorders

In neonates, these disorders usually present with jaundice, with or without anaemia, and abnormal red cell morphology on the blood film. Many cases of hereditary elliptocytosis present as an incidental finding in a neonate being investigated for a



(a) (b)

Figure 54.6 Red cell membrane disorders. (a) Blood film from a baby with hereditary pyropoikilocytosis showing microspherocytes, red cell fragments and polychromasia. (b) Blood film from a neonate with hereditary spherocytosis showing large numbers of spherocytes and polychromasia.

non-haematological disorder; anaemia is rare in heterozygotes and jaundice is often minimal. Most cases of hereditary spherocytosis do have jaundice in the neonatal period and moderate anaemia is fairly common. On the other hand, severe transfusion-dependent neonatal haemolytic anaemia due to an inherited red cell membrane disorder is very uncommon and typically due to hereditary pyropoikilocytosis which can be readily identified because of the characteristic low mean corpuscular volume (MCV) of 50–60 fL and bizarre red cell morphology. The main clues are a family history and an abnormal blood film, as red cell membrane disorders can nearly always be recognised by the characteristic shape of the red cells (Figure 54.6a). Identification of the exact type of membrane abnormality may require specialised investigations. The osmotic fragility test is of limited value in neonates and recent data indicate that the dye binding test is more useful. If the clinical phenotype is severe and family history or family studies are unhelpful, further investigation is useful to clarify the diagnosis and pattern of inheritance. This is mostly achieved by molecular methods, using next-generation sequencing panels targeting a number of genes known to be associated with red cell membranopathy. Ektacytometry, an automated method of demonstrating red cell osmotic fragility, is also used where available and can often be useful as a phenotypic readout of rare or new variants identified in the sequencing analyses.

Hereditary spherocytosis

This is the most common of the red cell membrane disorders to cause symptomatic anaemia, affecting 1 in 5000 live births to parents of northern European extraction. Hereditary spherocytosis is caused by mutations in the genes for *spectrin*, *ankyrin*, *protein 4.1* or *protein 3* and is usually inherited in an autosomal dominant fashion. About 10 to 15% cases of hereditary spherocytosis are inherited as a recessive disease due to bi-allelic mutations in genes encoding *protein 4.2*, *alpha-spectrin* or *ankyrin*. The blood film shows spherocytes and typically has an identical appearance to that of ABO haemolytic disease (Figure 54.6b). Neonatal anaemia due to hereditary spherocytosis is usually moderate (haemoglobin 70–100 g/L); it is not uncommon for affected neonates to require one or two transfusions during the neonatal period before a transfusion-free plateau haemoglobin of 80–100 g/L is achieved after a few months.

Hereditary elliptocytosis

This disorder is also caused by mutations in the *spectrin*, *ankyrin* or *protein 4.1* genes, but which have a different functional consequence to the mutations associated with hereditary spherocytosis. In neonates, hereditary elliptocytosis usually has no clinical manifestations apart from elliptocytes on the blood film. However, neonates who are homozygous or compound heterozygous for hereditary elliptocytosis mutations have severe haemolytic anaemia. The most common form is *hereditary pyropoikilocytosis*, which causes severe, persistent, transfusion-dependent haemolytic anaemia presenting in the neonatal period. The diagnosis is usually easily made from the low MCV and blood films of the baby (which show bizarre fragmented red cells and microspherocytes; see Figure 54.6a). Examination of the parental blood films is very helpful since one or both parents typically has common hereditary elliptocytosis, although silent *spectrin* mutations and de novo mutations may also occur and produce a severe phenotype in the baby despite apparently normal parental blood films. The treatment of neonates with hereditary pyropoikilocytosis is red cell transfusion. Transfusions should continue on a regular basis until the child is old enough to undergo splenectomy, to which there is an excellent response, with all patients rendered transfusion-independent.

Neonatal haemolysis due to red cell enzymopathies

The commonest inherited red cell enzymopathies presenting in the neonatal period are glucose-6-phosphate dehydrogenase (G6PD) deficiency and pyruvate kinase deficiency (see also Chapter 16).

G6PD deficiency

The prevalence of G6PD deficiency is particularly high in individuals from Central Africa (20%) and the Mediterranean (10%) but is also seen in the Indian subcontinent, the Far East and the Middle East. In neonates, G6PD deficiency nearly always presents with jaundice within the first few days of life; the vast majority of affected neonates are boys, as the *G6PD* gene is on the X chromosome. Neonatal jaundice due to G6PD deficiency is often severe, whereas anaemia is uncommon. The blood film is usually completely normal, but in the small number of neonates with anaemia due to G6PD deficiency, the blood film shows typical changes of oxidative haemolysis. The diagnosis is made by assaying G6PD on a peripheral blood sample. The pathogenesis of the jaundice is unclear, as most babies with G6PD deficiency have no evidence of haemolysis. Management of neonatal G6PD deficiency requires close monitoring of the bilirubin to prevent kernicterus, particularly when interactions with other risk factors for neonatal hyperbilirubinaemia are present, such as Gilbert syndrome, haemolytic disease of the newborn or hereditary spherocytosis, and also the counselling of the babies' parents regarding which medicines, chemicals and foods may precipitate haemolysis. G6PD deficiency does not cause chronic haemolysis (except in a few extremely rare G6PD variants) and therefore folic acid supplements are not indicated.

Pyruvate kinase deficiency

Although pyruvate kinase deficiency is the second most common inherited red cell enzymopathy in neonates, the estimated prevalence is only 50 cases/million, and therefore even large neonatal centres will only identify 1 case every 5 years. Pyruvate kinase deficiency is inherited in an autosomal recessive fashion. It is clinically heterogeneous, varying from anaemia severe enough to cause hydrops fetalis to a mild unconjugated hyperbilirubinaemia. In severe cases, the jaundice has a rapid onset within 24 hours of birth and exchange transfusion may be required. The diagnosis is made by measuring pretransfusion red cell pyruvate kinase activity where available. Mutation analysis of the *PKLR* gene that encodes this enzyme is increasingly used to confirm the diagnosis and provide genetic counselling if appropriate. In mild cases, enzyme activity may be relatively modestly reduced making the diagnosis difficult and it is often useful to assay levels in the parents for confirmation. The blood film is sometimes distinctive but more often shows non-specific changes of non-spherocytic haemolysis. Management in the neonatal period depends on the severity of the jaundice and anaemia. Some, but not all, children with pyruvate kinase deficiency are transfusion dependent and folic acid supplements should be given to prevent deficiency due to chronic haemolysis.

Other red cell enzymopathies

The other red cell enzymopathies are rare. The most important in the neonatal period is *triosephosphate isomerase deficiency*, as one-third of patients present with neonatal haemolytic anaemia, often many months before the devastating neurological features of this disorder become apparent. Another cause of acute haemolysis confined to the neonatal period is *infantile pyknocytosis*. This poorly understood condition, which is probably due to transient glutathione peroxidase deficiency, usually presents at 1–6 weeks of age. Infantile pyknocytosis is more common in preterm neonates and when it occurs in twins, both are usually affected. The anaemia associated with infantile pyknocytosis is often moderately severe, requiring one to two red cell transfusions before gradually resolving by the age of 2–3 months. The blood film typically shows changes of oxidative haemolysis during the neonatal period, but returns to normal as the haemolysis resolves (Figure 54.7) Measurement of glutathione peroxidase levels in affected neonates may be useful (parental levels are normal) but the diagnosis is usually easily made from the blood film. There are no reports of recurrence of haemolysis out-with the neonatal period in babies with infantile pyknocytosis and care should be taken to exclude other causes of haemolytic anaemia in any cases where the haemolysis does not resolve, or recurs, after the age of 3 months.

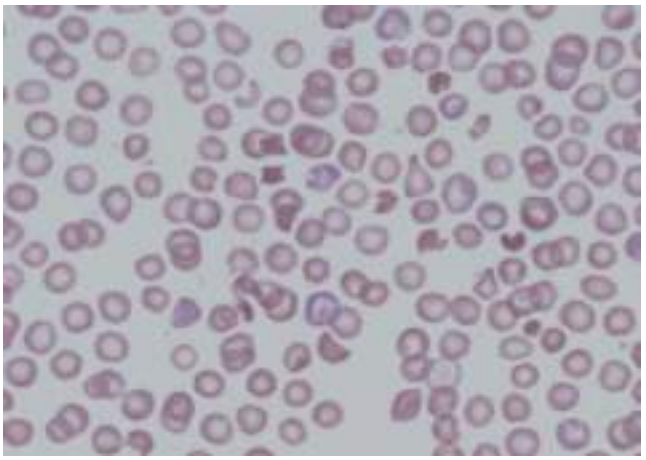


Figure 54.7 Infantile pyknocytosis. Blood film from a neonate with infantile pyknocytosis and a low level of erythrocyte glutathione peroxidase showing pyknocytes, fragmented red cells, polychromasia and occasional spherocytes.

Neonatal haemolysis due to haemoglobinopathies

The only haemoglobinopathy that typically presents in the neonatal period is α-thalassaemia major, as all four *α-globin* genes are deleted. Severe cases of HbH disease may also present with severe fetal or neonatal anaemia, although this is uncommon. Occasional non-thalassaemic structural *α- and γ-globin* gene mutations, which are clinically completely silent in adults and children, cause transient neonatal haemolytic anaemia in the neonate because they are unstable (e.g. Hb Hasharon, Hb Poole), whereas the major β-globin haemoglobinopathies (sickle cell disease and β-thalassaemia major) rarely manifest clinically in neonates.

α-Thalassaemia major

This predominantly affects families of Southeast Asian, Mediterranean or Middle Eastern origin, and presents with second-trimester fetal anaemia or hydrops fetalis, which is almost always fatal within hours of delivery. The only long-term survivors of α-thalassaemia major are those who have received intrauterine transfusions followed by regular postnatal transfusions and/or a bone marrow transplant. There is also a high incidence of hypospadias and limb defects in survivors, and others have severe neurological problems. If intrauterine transfusions are delayed until anaemia is severe, neonatal pulmonary hypoplasia is a cause of early mortality. The diagnosis of α-thalassaemia major should be suspected in any case of severe fetal anaemia that presents in the second trimester, and any case of hydrops fetalis with severe anaemia in which the parents come from high-prevalence areas, particularly Southeast Asia. Checking the blood counts of the parents will immediately identify whether they are at risk of having a child with α-thalassaemia major: both parents will have hypochromic microcytic red cell indices, with MCV usually below 74 fL and mean corpuscular haemoglobin (MCH) usually less than 24 pg. Prenatal diagnosis and genetic counselling are offered to all pregnant women at risk of having a baby with α-thalassaemia major. In countries with high prevalence of α-thalassaemia major but limited access to prenatal genetic diagnosis, screening is also done by early trimester ultrasound scan to detect fetal anaemia. The diagnosis of α-thalassaemia major is confirmed by DNA analysis that demonstrates deletions in all four genes encoding α-globin. Haemoglobin electrophoresis or HPLC from the affected fetus or infant shows Hb Barts, Hb Portland and sometimes HbH; HbF and HbA are absent); the blood film shows hypochromic microcytic red cells with vast numbers of circulating nucleated red cells (Figure 54.8).

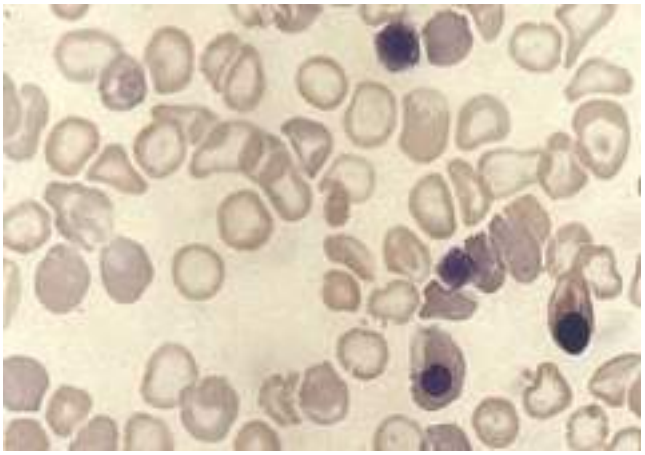


Figure 54.8 α-Thalassaemia major. Blood film from a neonate with α-thalassaemia major, born at 28 weeks' gestation, showing severe hypochromia, microcytosis, target cells, polychromasia and nucleated red cells.

Neonatal anaemia due to blood loss

Blood loss as a cause of neonatal anaemia may be very obvious (e.g. a cephalhaematoma or rupture of the cord) or the blood loss may be concealed and easy to miss unless specifically sought (e.g. fetomaternal bleeds). Usually, the most serious blood loss occurs prior to delivery and important causes of this are twin-to-twin transfusion and fetomaternal blood loss.

Twin-to-twin transfusion

This occurs in monochorionic twins with monochorial placentas where there are two amniotic sacs (diamniotic). Twin-to-twin transfusion syndrome carries a mortality of up to 80% without treatment and 15–40% with treatment. In most cases, bleeding is chronic, which usually results in a marked difference in birth weight between the twins. The donor twin is smaller, pale and lethargic and may have overt cardiac failure. By contrast, the recipient twin is often plethoric, with hyperviscosity, hyperbilirubinaemia, polycythaemia and, in the most severe cases, disseminated intravascular coagulation (DIC). Where twins survive to delivery, the difference in Hb between donor and recipient twin where there has been chronic blood loss is usually <50 g/L. Bleeding may also occur acutely during labour, with signs similar to those of fetomaternal haemorrhage. Early detection can improve outcomes in twin-to-ttwin transfusions. This is undertaken by ultrasound screening and fetoscopic laser surgery to stop aberrant placental blood flow.

Fetomaternal haemorrhage

Most significant fetomaternal bleeds occur during the third trimester, either spontaneously or secondary to trauma and involve very small quantities of blood (0.5 mL or less). Much more of a clinical problem are acute fetomaternal bleeds where there is loss of more than 20% of the blood volume. This may cause intrauterine death or the baby may be born with hydrops or circulatory shock. In some cases, the baby appears well, but pale, at birth and collapses a few hours later. Diagnostic clues are anaemia at birth in an otherwise well baby with no or minimal jaundice. The most useful diagnostic tests are a Coombs test to exclude immune haemolysis, a reticulocyte count to exclude red cell aplasia, a Kleihauer test on maternal blood to quantify the number of HbF-containing fetal red cells in the maternal circulation and a blood film (Figure 54.9). In RhD-negative women, accurate quantification of fetal blood in maternal circulation is undertaken by flow cytometry so that the appropriate dose of anti-D can be administered to prevent alloimmunisation. Quantification of fetal blood loss in this manner is not routinely available to RhD-positive women, although a case may be made in its support as it will assist the neonatal team in managing the baby. Blood loss around the time of delivery is usually due to obstetric complications (e.g. placenta praevia, placental abruption); in these circumstances, the babies are often extremely ill, with circulatory shock, anaemia worsening rapidly after birth, large numbers of circulating nucleated red cells and DIC.

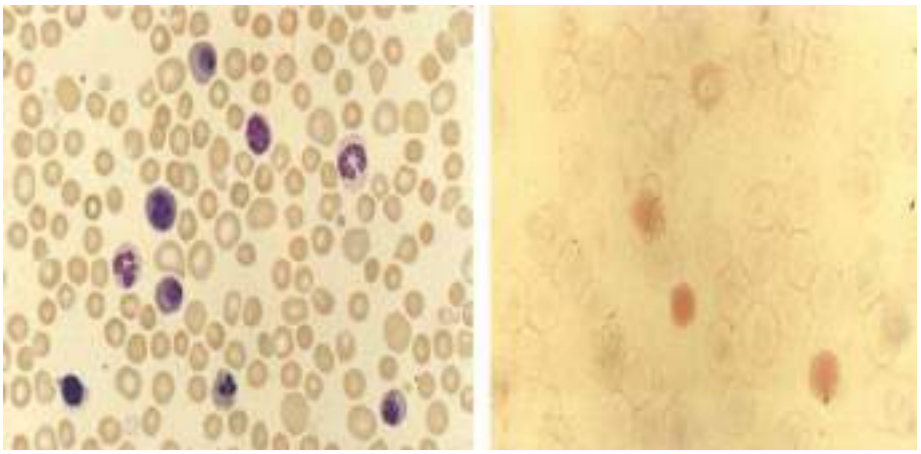

(a) (b)

Figure 54.9 Fetomaternal haemorrhage. (a) Blood film from a neonate with a haemoglobin of 50 g/L at birth due to a large fetomaternal haemorrhage; the main features are the marked polychromasia, large numbers of nucleated red cells and normal red cell morphology. (b) Kleihauer test showing HbA-containing maternal 'ghost' cells and pink-staining HbF-containing fetal cells.

Anaemia of prematurity

The normal fall in haemoglobin in preterm neonates has been termed 'physiological anaemia of prematurity', as it does not appear to be associated with any abnormalities in the baby. The pathogenesis is not fully elucidated, but contributory factors include the reduced lifespan of fetal erythrocytes, the relatively low erythropoietin concentration, the rapid growth rate and iatrogenic blood loss. Routine supplementation of preterm neonates with folic acid and iron means that nutritional deficiency rarely plays a role. The diagnosis is usually straightforward: a well-preterm baby with a slowly falling haemoglobin, unremarkable blood film showing normochromic/normocytic red cells, slightly low reticulocytes (20×10^9/L) and no nucleated red cells.

Management of anaemia of prematurity and the role of erythropoietin

The severity of anaemia of prematurity and thereby the need for red cell transfusion can be reduced by: (i) limiting iatrogenic blood loss by appropriate use of blood tests, (ii) administering iron and folate supplements to all preterm infants (iron 3 mg/kg daily from 4–6 weeks of age or iron-fortified formula with 0.5–0.9 mg/dL of iron and folic acid 50 µg daily or 500 µg once weekly) and (iii) judicious use of erythropoietin. The many controlled trials of recombinant erythropoietin for prevention of neonatal anaemia have been reviewed extensively. Erythropoietin reduces the number of transfusions in relatively well infants with low transfusion requirements, but not in sick preterm infants who have a need for frequent phlebotomy and multiple transfusions. The main roles for recombinant erythropoietin in neonates are in ameliorating the anaemia in infants who have received intrauterine transfusions for alloantibody-mediated anaemia and in preterm babies of Jehovah's Witnesses. The usual dose is 300 µg/kg of epoetin-β by subcutaneous injection three times per week, starting in the first week of life, together with oral iron supplements.

Indications for red cell transfusion in neonatal anaemia

The BSH guidelines for transfusion of fetuses, neonates and older children contain recommendations about the products and indications for red cell transfusion in neonates. These are consensus guidelines and need to be adapted for use in each individual neonatal intensive care unit, depending on the case mix of babies, as adherence to strict neonatal transfusion guidelines reduces both the number of transfusions and donor exposure.

A simple diagnostic approach to neonatal anaemia

Red cell disorders associated with neonatal or fetal anaemia present in three main ways: with a low haemoglobin, with jaundice due to haemolysis or with hydrops. A diagnostic algorithm to help identify which of these causes is most likely, which can be excluded and what further investigations are most appropriate is shown in Figure 54.2.

Neonatal polycythaemia

For both term and preterm infants, polycythaemia can be defined as a central venous haematocrit of greater than 0.65, as there is an exponential rise in blood viscosity above this level. However, even at haematocrits greater than 0.70, only a minority of neonates have clinical signs of hyperviscosity, such as lethargy, hypotonia, hyperbilirubinaemia and hypoglycaemia. Causes of polycythaemia are shown in Table 54.3. Treatment is controversial and is not necessary for infants with very minor

Table 54.3 Causes of neonatal polycythaemia.

- Intrauterine growth restriction
- Maternal hypertension
- Maternal diabetes
- Chromosomal disorders: trisomy 21, 18 or 13
- Twin–twin transfusion
- Delayed clamping of the cord
- Endocrine disorders: thyrotoxicosis, congenital adrenal hyperplasia

symptoms (e.g. borderline hypoglycaemia or poor peripheral perfusion). Infants with neurological signs and a haematocrit greater than 0.65 should have a partial exchange transfusion (using a crystalloid solution such as normal saline) to reduce the haematocrit to 0.55.

White cell disorders

Normal values

In the neonate, normal values for leucocytes, particularly neutrophils, are affected by a number of factors including gestational age, postnatal age, antenatal history, perinatal history and ethnic origin. Neutrophil counts in healthy babies increase for the first 12 hours and then fall to a nadir at 4 days of age. The neutrophil count is higher in capillary samples and after vigorous crying; it is lower in neonates of African origin. Healthy preterm babies often have circulating myeloblasts and lymphoblasts, although these usually form less than 5% of the white cell differential count.

Neutropenia

A pragmatic approach is to consider a neutrophil count at birth of less than 2×10^9/L as abnormal and worth monitoring, and a neutrophil count during the first month of life of less than 0.7×10^9/L as significant enough to merit further investigation.

Causes of neutropenia

The commonest cause of neutropenia at birth in preterm neonates is reduced neutrophil production, secondary to intrauterine growth restriction or maternal hypertension. Most affected neonates also have thrombocytopenia and increased erythropoiesis (polycythaemia and/or increased circulating nucleated red cells). The underlying mechanism for these haematological abnormalities is chronic fetal hypoxia. The neutropenia resolves spontaneously usually within a few days of birth and does not persist beyond the first 2 weeks of life. The commonest cause of neutropenia in term infants is bacterial or viral infection. Infection-associated neutropenia is also self-limiting and therefore persistent neutropenia in a term or preterm baby should always be investigated. Other important causes of neutropenia are alloimmune neutropenia and severe congenital neutropenia (SCN) due to failure of neutrophil production (e.g. Kostmann syndrome), both of which predispose to severe neonatal infection.

Alloimmune neutropenia

This is the neutrophil equivalent of haemolytic disease of the newborn and alloimmune thrombocytopenia. Alloimmune neutropenia occurs when fetal neutrophils express paternally derived neutrophil-specific antigens absent on maternal neutrophils and against which the mother produces IgG neutrophil alloantibodies. The causative antibodies are usually anti-HNA1 or -HNA2. Severe cases present in the first few days of life with fever and infections of the respiratory tract, urinary tract and skin, particularly due to *Staphylococcus aureus*, and the mainstay of treatment is antibiotics. The diagnosis is made by demonstrating antineutrophil antibodies in the mother and baby, which react against paternal, but not maternal, neutrophil antigens. The neutropenia is self-limiting, usually resolving within 1–2 months. Since alloimmune neonatal neutropenia in everyday clinical practice is very uncommon, and yet case series show that it affects 3% of all deliveries, it is likely that most milder cases are missed as they do not present with clinical problems and routine full blood counts are not performed on well babies.

Congenital and inherited neonatal neutropenias

Although rare, it is important to identify SCN in the neonatal period, since such babies are at risk of life-threatening infection from birth. Investigations are indicated where neutropeneutropenia persists beyond the first 2 weeks of life, despite resolution of infection, if there is a relevant family history or consanguinity, if the neutropenia is severe ($< 0.5 \times 10^9$/L) or if the baby has typical dysmorphic features (e.g. thumb/radial abnormalities in Fanconi anaemia). SCN is the most likely cause in the neonatal period. Infants usually present with severe infections and a marked neutropenia ($<0.2 \times 10^9$/L), often with a compensatory monocytosis. The diagnosis is made by the severity of the neutropenia, the bone marrow appearance (arrest of differentiation at the myelocyte/promyelocyte stage) and the absence of antineutrophil antibodies. The inheritance of SCN can be autosomal recessive or dominant or X-linked. The molecular basis of most cases is increasingly being identified both through classical linkage studies and gene discovery approaches. The causative genes are important for normal myeloid homeostasis, including neutrophil differentiation, survival and function. Mutations in *ELA2* (neutrophil elastase) are the most common cause of SCN, but other causes include mutations in *GFI1* (growth factor-independent protein 1), *WAS* (Wiskott–Aldrich syndrome protein, WASp), *JAGN1* and several genes affecting the endosomal–lysosomal system, such as *AP3B1, LAMTOR2, VPS13B* and *VPS45*. In 2007 biallelic mutations in the *HAX1* gene were found to explain the SCN in

the original Kostmann family first described in 1956, but this appears to be a rare cause of the disease.

Congenital leukaemias

Congenital leukaemia is rare, affecting ~5 neonates/per million live births. The most common form of congenital leukaemia is the transient leukaemia seen in neonates with Down syndrome, up to 10% of which will be affected. In children without Down syndrome, the majority of congenital leukaemias are associated with rearrangement of the *MLL* gene and manifest as acute lymphoblastic leukaemia (ALL) in 60% of cases and acute myeloid leukaemia (AML, typically monoblastic) in the remaining 40%.

Leukaemia in neonates with Down syndrome

Around 5–10% of neonates with Down syndrome have a transient megakaryoblastic leukaemia, also known as transient abnormal myelopoiesis (TAM) or transient myeloproliferative disorder (TMD), characterised by leucocytosis and circulating blast cells (Figure 54.10). TAM develops in fetal life and nearly always presents in the first week of life. Most cases spontaneously resolve within 2–3 months and no treatment is indicated. In severe cases, (10–20%) neonates develop progressive disease within days or weeks of birth due to blast cell infiltration of the liver in association with liver fibrosis. In such cases pleural/pericardial effusions, ascites, hepatomegaly, liver dysfunction and coagulopathy are common and treatment with low-dose cytosine arabinoside is often successful. TAM is unique to neonates with Down syndrome and is now known to be caused by the effects of acquired mutations in the haemopoietic transcription factor gene *GATA1* in fetal liver haemopoietic cells. Acquired *GATA1* mutations are now known to affect almost 30% of all neonates with Down syndrome, but only half of these will manifest with clinical or haematological signs; the remaining cases are clinically silent because the mutant *GATA1* clones are very small. In most cases, the mutant *GATA1* clones disappear over the first few months of life, as all signs of TAM disappear. In up to 10% of neonates with Down syndrome who have TAM or silent *GATA1* mutations, an acute megakaryoblastic leukaemia (DS-AMKL) develops before the age of 5 years, usually between 12 and 18 months of age. In these cases, the TAM usually appears to resolve, but this is followed several months later by myelodysplasia, which subsequently evolves to acute leukaemia. DS-AMKL, unlike TAM, is not transient and must be treated with chemotherapy when the outcome is usually very good, with 80–90% long-term survival. Since the same *GATA1* mutations are present both in TAM and DS-AMKL, this indicates that the leukaemia arises from a mutant *GATA1* clone present at birth. Mutations in the cohesin complex genes have been shown in several studies to be the most common mechanism responsible for transformation of TAM to DS-AMKL.

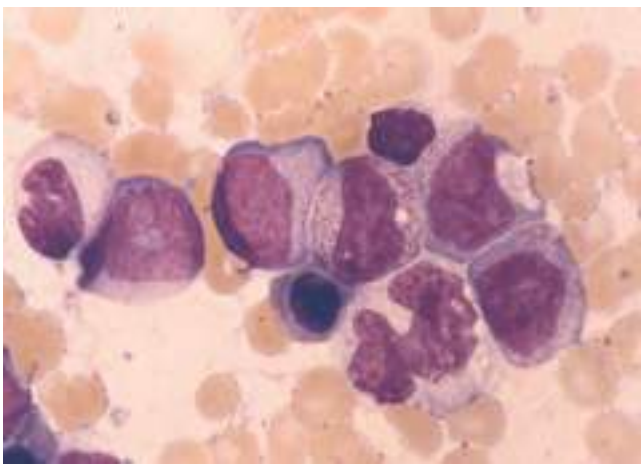


Figure 54.10 Transient leukaemia in a neonate with Down syndrome. Leucoerythoblastic blood film showing increased numbers of blast cells, which spontaneously returned to normal by 2 months of age.

Leukaemia in children without Down syndrome

Affected babies present, often within hours of birth, with signs of anaemia, thrombocytopenia and/or skin lesions caused by leukaemic infiltration. Congenital leukaemia with nodular skin deposits of leukaemic cells is one cause of the classical 'blueberry muffin' rash in a newborn baby. Some cases present *in utero* with intrauterine death or hydrops. CNS leukaemia in a neonate may present as a bulging fontanelle. The blood film and bone marrow aspirate show large numbers of primitive blast cells. The most common cytogenetic abnormalities are translocations involving 11q23 resulting in rearrangement of the *MLL* gene and t(1;22), which is associated with the *OTT::MAL* fusion gene and an aggressive form of acute megakaryoblastic leukaemia. The prognosis is extremely poor (~20% long-term survival); few are cured by chemotherapy and bone marrow transplantation may be the best option.

Haemostasis and thrombosis in the newborn

Bleeding and thrombotic problems are relatively common in neonates, particularly in those who are preterm and/or sick. The vast majority of bleeding problems are acquired and secondary to perinatal complications, including perinatal asphyxia and severe infection. Inherited bleeding disorders, with the exception of haemophilia A and B, are rare in the newborn. By contrast, there has been a marked increase in the identification of inherited thrombophilias in neonates and the most challenging aspect of this has been understanding their significance, both for management of the neonate and for genetic counselling.

Developmental haemostasis

Coagulation proteins are present at measurable levels from the 10th week of gestation and gradually rise during fetal life. They do not cross the placenta, or do so in very small amounts, and therefore need to be independently synthesised by the fetus. 'Normal values' in the neonate vary not only with gestational age, but also with postnatal age. Data for babies at less than 30 weeks' gestation derive from fetoscopy samples: levels of the vitamin-K-dependent factors (FII, FVII, FIX and FX) and of FXI and FXII are all low (<40% of adult values) and remain so during the first month of life. In contrast, even in preterm babies (>30 weeks gestation at birth), levels of FV, FXIII and fibrinogen are normal at birth and levels of FVIII and von Willebrand factor (VWF) are normal or increased.

Platelet counts at birth in term and preterm neonates are within the normal adult range. However, increasing evidence points to significant differences in platelet production during fetal life. Fetal megakaryocytes are smaller and of lower ploidy despite being more proliferative in *ex-vivo* cultures than adult megakaryocytes and are more sensitive to thrombopoietin. Interestingly, in response to consumptive thrombocytopenia, neonates increase the number of megakaryocytes rather than megakaryocyte size consistent with fundamental differences in regulation of platelet production in the fetus and newborn compared to later in life. These differences may help to explain why many studies have found impaired *in vitro* function of neonatal platelets. The most consistent abnormalities are reduced aggregation in response to adrenaline, ADP and thrombin. Their significance in clinical practice is still not clear, as the bleeding time is normal in term and preterm infants (≤135 s).

Screening tests for bleeding disorders

Nearly all significant bleeding disorders in neonates can be identified using simple screening tests; exceptions are FXIII deficiency and platelet function disorders. It is often helpful to test both parents for coagulation abnormalities to help identify inherited disorders as there is considerable overlap between coagulation factor levels in inherited or acquired deficiency states and neonatal normal ranges, particularly in preterm neonates. In addition, it is advisable to repeat investigations for the levels of coagulation factors at the age of 6–12 months to confirm (or exclude) inherited deficiencies. The most useful screening tests in neonates are the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen and platelet count. Bleeding times are generally unhelpful in neonates and investigation of platelet function abnormalities is often deferred until a few months of age when platelet aggregometry becomes practical.

Inherited coagulation disorders

The commonest inherited disorders presenting in the neonatal period are FVIII deficiency (haemophilia A), which has a frequency of 1 in 5000 male births, and FIX deficiency (haemophilia B), which occurs in 1 in 30,000 male births.

Factor VIII deficiency

Almost 40% of patients with inherited FVIII deficiency present in the neonatal period. The clinical signs include intracranial haemorrhage, cephalohaematomas and bleeding after circumcision or from venous or arterial puncture sites. As in adults, the diagnosis is made by finding an isolated prolonged APTT and reduced FVIII clotting activity. Acute management of the bleeding neonate with haemophilia requires intravenous administration of recombinant FVIII (50–100 units/kg twice daily) to achieve FVIII levels of 1.0 unit/mL. As the half-life of FVIII is shorter than in adults, more frequent dosing or a continuous FVIII infusion may be required. For neonates with intracranial bleeding, treatment with FVIII should continue for at least 2 weeks. Fibrin glue may be useful in circumcision-associated bleeds. The optimal mode of delivery for carriers known to be carrying an affected child or at risk of carrying an affected child remains controversial. However, there is general agreement that instrumental delivery, fetal blood sampling and use of fetal scalp electrodes should be avoided. Where available, non-invasive prenatal testing of cell-free fetal DNA in maternal serum is sometimes used for sex determination while late amniocentesis can be used to identify unaffected fetuses where delivery can safely proceed without intervention. The role of prophylactic FVIII administration to affected newborns following difficult delivery to reduce the risk of intracranial bleeding (1–4%) is controversial but is increasingly used. Prophylactic FVIII should also be used for an affected newborn when a previous sibling has had a major intracranial bleed.

Factor IX deficiency

Deficiency of FIX is clinically indistinguishable from FVIII deficiency. As FIX levels are also low in liver disease and vitamin K deficiency, it is important to recheck FIX levels at 6 weeks and 6 months of age if the diagnosis is in doubt. Neonates with bleeding are treated with recombinant FIX concentrate (100 units/kg IV once daily, monitored to achieve a FIX level of 1.0 IU/mL).

von Willebrand disease in neonates

The two forms of von Willebrand disease (VWD) that can present in neonates are type 2B vWD and type 3 VWD. Type 2B disease presents with thrombocytopenia and bleeding and is very uncommon. Type 3 vWD has a clinical phenotype similar

to haemophilia, as levels of both VWF and FVIII are low. The inheritance in type 3 vWD is autosomal recessive. The diagnosis is made by measuring VWF, FVIII and the pattern of VWF multimers. At present, type 3 vWD is treated with a high-purity VWF:FVIII concentrate (such as Wilate®).

Factor XIII deficiency

This rare autosomal recessive disorder usually presents with delayed bleeding from the umbilical cord during the first 3 weeks of life. The diagnosis is made by measuring clot solubility in 5 mol/L urea solution as a screening test, followed by a specific FXIII assay. Molecular tests for the common FXIII mutations are also available. The routine diagnostic coagulation screen is normal in Factor XIII deficiency. Bleeding is treated with FXIII concentrate.

Acquired disorders of coagulation

Causes of acquired coagulopathy in neonates include vitamin K deficiency, DIC, liver disease, metabolic disorders, extracorporeal membrane oxygenation (ECMO) and giant haemangioendotheliomas (Kasabach–Merritt syndrome).

Vitamin K deficiency

Levels of vitamin-K-dependent procoagulant factors (FII, FVII, FIX and FX), protein C and protein S are low at birth because of poor placental transfer of vitamin K, low vitamin K stores at birth, low vitamin K in breast milk and the lack of bacterial vitamin K synthesis in the sterile neonatal gut. Vitamin K deficiency can lead to haemorrhagic disease of the newborn, also known as vitamin K deficiency bleeding (VKDB). Early VKDB presents in the first 24 hours of life with severe gastrointestinal and intracranial haemorrhage. It is usually due to maternal medication that interferes with vitamin K, for example, anticonvulsants (phenobarbital, phenytoin), antituberculous therapy or oral anticoagulants. Classical VKDB presents at 2–7 days in 0.25–1.7% of babies who have not received prophylactic vitamin K at birth, especially if breastfed or with poor oral intake. Late VKDB occurs between 2 and 8 weeks after birth and presents with sudden intracranial haemorrhage in an otherwise well, breastfed term baby or in babies with liver disease.

The diagnosis of VKDB is usually made by an isolated prolonged PT, which corrects following vitamin K administration. If doubt remains, assays of the inactive form of FII (decarboxyprothrombin; PIVKA II) can be used to confirm the diagnosis. Treatment of VKDB is vitamin K 1 mg intravenously or subcutaneously with fresh-frozen plasma only in severe haemorrhage. Classic and late VKDB can be prevented by a single intramuscular dose of vitamin K or, slightly less effectively, by weekly 1-mg oral doses of vitamin K over the first 12 weeks of life (in exclusively breast-fed infants neither single oral dose of vitamin K nor daily very low-dose vitamin K 25 μg are effective in preventing classic VKDB). Some studies, but not others, suggest a link between intramuscular vitamin K at birth and later childhood malignancies. In healthy babies, the choice of which route of administration is increasingly being left to parents. However, infants of mothers taking drugs that inhibit vitamin K (e.g. warfarin) are at risk of early VKDB and these babies should receive intramuscular vitamin K 1 mg as soon as possible after birth.

Disseminated intravascular coagulation

The main triggers of DIC in neonates are severe hypoxia and/or acidosis and sepsis. The main causes are DIC secondary to acute perinatal hypoxia (for example in association with placental abruption or a large, acute fetomaternal haemorrhage), severe sepsis and necrotising enterocolitis. DIC may also be seen secondary to perinatal Coxsackie virus infection. Neonates with DIC are nearly always very sick and the clinical signs include generalised bleeding, pulmonary haemorrhage and oozing from venepuncture sites. The usual pattern of coagulation abnormalities in neonatal DIC is prolongation of the PT, APTT and TT, together with low platelets and fibrinogen. D-dimers are increased, but are not specific and can be found in healthy neonates with no evidence of coagulopathy. The most important aspect of management of DIC is treatment of the underlying cause. Blood product replacement is indicated for clinical bleeding, aiming to maintain the platelet count above 30×10^9/L, normalisation of PT and APTT and fibrinogen greater than 1 g/L.

Neonatal thrombocytopenia

Thrombocytopenia occurs in 1–5% of all neonates and in up to 50% of neonates who are preterm and sick. It usually presents in one of two clinical patterns: early onset thrombocytopenia (within 72 hours of birth) and late-onset thrombocytopenia (after 72 hours of life) (Table 54.4). The most frequent causes of early thrombocytopenia in preterm infants are intrauterine growth restriction and maternal hypertension or diabetes; the most frequent causes in term infants are neonatal alloimmune thrombocytopenia (NAIT) and thrombocytopenia secondary to maternal immune thrombocytopenic purpura (ITP). The most common causes of late thrombocytopenia are sepsis and necrotising enterocolitis.

Neonatal alloimmune thrombocytopenia

NAIT is the platelet equivalent of haemolytic disease of the newborn and affects around 1 in 1000 pregnancies. It is frequently severe (platelet count $<30 \times 10^9$/L) and occurs in the first pregnancy in almost 50% of cases. Thrombocytopenia results from transplacental passage of maternal platelet-specific antibodies to human platelet antigens (HPA), which the mother lacks, but

Table 54.4 Causes of neonatal thrombocytopenia.

Early onset (<72 hours)
- Placental insufficiency (PET, IUGR, diabetes)
- Neonatal alloimmune thrombocytopenia
- Birth asphyxia
- Perinatal infection (group B *Streptococcus*, *Escherichia coli*, *Listeria*)
- Congenital infection (CMV, toxoplasmosis, rubella, Coxsackie)
- Maternal autoimmune (ITP, SLE)
- Severe Rh- antigen associated HDN
- Thrombosis (renal vein, aortic)
- Aneuploidy (trisomy 21, 18, 13)
- Congenital/inherited (TAR, Wiskott–Aldrich)

Late onset (>72 hours)
- Late-onset sepsis and necrotising enterocolitis
- Congenital infection (CMV, toxoplasmosis, rubella)
- Maternal autoimmune (ITP, SLE)
- Congenital/inherited (TAR, Wiskott–Aldrich)

CMV, cytomegalovirus; HDN, haemolytic disease of the newborn; ITP, idiopathic thrombocytopenic purpura; IUGR, intrauterine growth restriction; PET, pre-eclampsia; SLE, systemic lupus erythematosus; TAR, thrombocytopenia with absent radii.

which the fetus inherits from the father. In 80% of cases amongst Caucasian women in whom antibodies are detectable, these are anti-HPA-1a antibodies and in 10–15% anti-HPA-5b; occasional cases are due to anti-HPA-3a. HLA-DRB3* 0101-positive women are 140 times more likely to make anti-HPA-1a than HLA-DRB3* 0101-negative women. Intracranial haemorrhage occurs in 10% of cases, with long-term neurodevelopmental sequelae in 20% of survivors. The diagnosis is made by demonstrating platelet antigen incompatibility between mother and baby and anti-HPA antibodies in the mother. Where possible the neonate and both parents should have platelet genotyping performed to determine their HPA alloantigen status: HPA-1a, -2, -3, -5b and -15 are the most common genotypes in Caucasian women and account for 95% cases while NAIT due to HPA-4b, 6b and 21b is more common in Asian families. Where a clinical diagnosis of NAIT is made and no antibodies or maternal–neonatal platelet incompatibility to the most common HPA antigens has been excluded, it is not usually possible to identify the causal platelet antigen. Very few of these unexplained cases are due to antibodies against minor HPA (such as HPA-6w and HPA-9w) and, therefore, most groups currently do not routinely screen for these very low-frequency HPA antigens, except in selected severe cases.

Management of NAIT

Management depends on the gestation and postnatal age of the baby, the history of previously affected siblings, the platelet count and the extent of any bleeding. All neonates suspected to have NAIT should have a cranial ultrasound scan to exclude the presence of an ICH. Since the platelet count usually falls over the first 4–7 days of life, all thrombocytopenic neonates with NAIT should be monitored until there is a sustained rise in their platelet count into the normal range. Platelet transfusion is not indicated in a term neonate with platelet counts $>30 \times 10^9$/L who has no evidence of bleeding. Transfusion with HPA-compatible (usually HPA1a, 5b-negative) platelets is recommended for:
- platelet counts below 30×10^9/L in term neonates who have no evidence of bleeding;
- platelet counts below 50×10^9/L and an ICH and/or a previously affected sibling with an ICH and/or birthweight < 1000 g.

Neonates who have received intrauterine platelet transfusions should be given irradiated platelets to prevent transfusion-associated graft-versus-host disease. In some cases, thrombocytopenia may persist for up to 8–12 weeks. In such cases, intravenous IgG (total dose 2 g/kg, over 2–5 days) may be useful to reduce the need for repeated platelet transfusions. Antenatal management of NAIT depends on the severity of previously affected neonates, the HPA genotype of the father and on the HPA antibody specificity. Most centres have abandoned the invasive approaches based on fetal blood sampling plus fetal transfusion with HPA-compatible platelets as this carries a risk of preterm delivery and fetal death. Instead, most centres now use a non-invasive approach relying on maternal intravenous IgG therapy during the second half of the pregnancy.

Neonatal autoimmune thrombocytopenia

The majority of cases are secondary to transplacental passage of maternal platelet autoantibodies in maternal ITP, which affects 1–2 in 1000 pregnancies. Most of the remaining cases are secondary to maternal systemic lupus erythematosus (SLE) although neonatal Kawasaki disease has also been reported. Around 10% of infants develop thrombocytopenia, of which around half will develop platelets $<20 \times 10^9$/L. The risk of major haemorrhage, including ICH, is estimated at ~1%. In babies with severe thrombocytopenia, intravenous IgG (400 mg/kg/day given over 2–4 hours for 5 days or 1 g/kg/day for 2 days, total dose 2 g/kg) is usually effective. In some cases, neonatal thrombocytopenia is prolonged and may last up to 12 weeks before spontaneously resolving.

Indications for platelet transfusion

A number of countries have published consensus guidelines to help decide the indications for platelet transfusion in term and preterm neonates. In general, platelet transfusion is not considered necessary in well babies if the platelet count is above $20–30 \times 10^9$/L. Most groups agree that the threshold for transfusing sick babies, particularly preterm babies in the first few weeks of life, should be higher ($30–50 \times 10^9$/L).

Neonatal thrombosis: physiology and developmental aspects

Neonates have an increased risk of thrombosis (2.4 per 1000 hospital admissions) compared with older infants and children. This is largely due to the physiologically low levels of many of the inhibitors of coagulation and the frequent use of indwelling vascular catheters. Concentrations of antithrombin, heparin cofactor II and protein C at birth are 30–50% of adult values; protein S levels are also low, but overall protein S activity is normal as it exists mainly in its free active form due to the virtual absence of its binding protein (C4b-BP) in neonates. Levels of plasminogen at birth are only 50% of adult values so neonates have a reduced ability to generate plasmin in response to fibrinolytic agents.

Screening tests for thrombophilia in neonates

The only inherited deficiencies for which there is a proven role in neonatal thrombosis are deficiencies in proteins C and S, which cause purpura fulminans, and deficiency of antithrombin, although presentation in the neonatal period is extremely rare. FV Leiden and the prothrombin 20210A promoter mutation (prothrombin^{20210A}) have not yet been reported to cause neonatal thrombotic problems in isolation. The role of thrombophilia screening in neonates is controversial, since the significance of any findings remains very difficult to assess, particularly since the risk of recurrence of thrombosis after an episode in the neonatal period is very low (~3%). Guidelines from the Haemostasis and Thrombosis Task Force of the British Society for Haematology state that congenital thrombophilia should be considered and screened for in any child with:

- clinically significant thrombosis, including spontaneous thrombotic events, unanticipated or extensive venous thrombosis, ischaemic skin lesions or purpura fulminans and
- a positive family history of neonatal purpura fulminans

The screening tests that should be performed in all suspected cases of thrombophilia include protein C activity, protein S, antithrombin, FV Leiden and prothrombin ^{20210A}. In addition, babies with thrombosis born to mothers with SLE and/or antiphospholipid syndrome should be tested for lupus anticoagulant, as antiphospholipid antibodies may cross the placenta and are a rare cause of neonatal thrombosis in such babies. The relevance of serum liporotein a and the MTHFR genotype to neonatal management is unclear at present.

Inherited thrombotic disorders in neonates

Proteins C and S deficiency

Protein C deficiency occurs in 1 in 160,000–360,000 births. Affected babies usually present within hours or days of birth with purpura fulminans, in which there is DIC and rapidly progressive life-threatening haemorrhagic necrosis due to dermal vessel thrombosis or cerebral, renal vein or ophthalmic thrombosis. The diagnosis of protein C deficiency is made by the clinical picture and undetectable levels of protein C (<0.01 U/mL) in the patient, together with heterozygote levels in the parents. Protein-C-deficient heterozygotes rarely present as neonates and have low protein C levels, which may overlap with the lower limit of normal in the first few months of life, delaying diagnosis until 6 months or later. Treatment is with protein C concentrate (40 U/kg, aiming to maintain a plasma level >0.25 U/mL). Protein S deficiency presents with identical features; levels of protein S are undetectable (<0.01 U/mL) and treatment is with fresh-frozen plasma (10–20 mL/kg) to maintain a plasma protein S in excess of 0.25 U/mL. The rarity of thrombosis in neonates with protein S deficiency may in part reflect the relatively high level of active protein S because of the undetectable levels of C4b-binding protein. Patients are usually managed in specialised haemophilia centres and confirmatory genetic diagnosis by next-generation sequencing is increasingly being performed in these rare conditions.

Antithrombin deficiency

Homozygous antithrombin deficiency usually presents later in childhood, but neonatal deep venous thrombosis and inferior vena cava thrombosis have been reported. Heterozygous antithrombin deficiency is more common (1 in 2000–5000 births); it usually presents in the second decade of life, but neonatal presentation with aortic thrombosis, myocardial infarction and cerebral dural sinus thrombosis may occur.

Acquired thrombotic problems in neonates

The most common risk factors for neonatal thrombosis are the presence of an intravascular catheter and shock, secondary to sepsis, hypoxaemia or hypovolaemia. Maternal factors, such as maternal thrombophilia, alcohol and cocaine use and preeclampsia, may also increase the risk of neonatal thrombosis. Thrombosis particularly affects the renal veins, the aorta, aortic arch or cerebral vessels. Catheter-related thrombosis causes more than 80% of venous thromboses and more than 90% of arterial thromboses. The diagnosis is made by Doppler ultrasound or contrast angiography. Treatment of catheter-related thrombosis depends on the severity and extent of thrombosis. The first step is prompt removal, where possible. If signs progress despite catheter removal, unfractionated heparin or low-molecular-weight heparin should be started using a dosing regimen adapted for neonates. Thrombolytic therapy with urokinase or tissue plasminogen activator has been used successfully for catheter-related thrombosis in neonates, but experience in preterm neonates is very limited. It is important to maintain fibrinogen at levels greater than 1 g/L and the platelet count greater than 50×10^9/L in neonates receiving thrombolytic therapy, and heparin (starting dose 28 U/kg per hour) is often given to maintain vessel patency after thrombolytic therapy, although

there is no evidence that this is beneficial. Excellent guidelines for antithrombotic therapy in neonates are regularly updated by the American College of Chest Physicians.

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CHAPTER 55

Supportive care in the management of leukaemia

55

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Introduction

Supportive care significantly contributes to the success of therapy for leukaemia and other haematological malignancies. It is defined as a broad range of interventions that ameliorate the symptoms of a disease, the side effects of treatment, whilst also addressing psychological, cultural, social and spiritual factors. Traditionally, the main focus was reactive on restoring health (rehabilitation) but increasing the focus is on prevention and preparation for therapy (prehabilitation) to subsequently allow swifter post-treatment recovery.

For purpose of this chapter, we divide supportive care into:

- Psychological, social and financial aspects
- Reproductive aspects
- Blood count abnormalities
- Skin, nail, dental, auditory and visual complications
- Infections
- Metabolic complications
- Nutrition and physiotherapy
- Chemotherapy-induced nausea and vomiting (CINV)
- Pain control and palliation.

Psychological, social and financial aspects

Optimal healthcare for leukaemia involves active consideration of the psychological factors that can affect clinical outcomes, patient experience of care and quality of life.

It is important to evaluate and target psychological factors that can affect safe and optimal treatment. Psychological factors such as self-efficacy, health beliefs, health-related trust and trauma, are systematically related to the self-management behaviour that is required of patients – e.g. medication adherence, timely reporting of symptoms, engagement in rehabilitation, etc. Current advances in outpatient treatment pathways (e.g. ambulatory haematopoietic stem cell transplants) require psychological expertise in evaluating individual capabilities and risks, and stratifying care and pathways accordingly.

In relation to patient experience and quality of life, the aims of comprehensive healthcare are to prevent distress, provide personalised support and promote psychological adjustment.

The term 'psychological adjustment' refers to the continuous, iterative process of adaptation that is taking place psychologically as people move through the 'journey' of cancer diagnosis and treatment. The pre-existing 'mental models' that the person enters into this process with (for instance, assumptions about independence, control, physical integrity and an open future) will be challenged by a multitude of intense and unpredictable physical and psychosocial experiences. Coping with and processing these adversities and re-constituting one's 'mental models' to fit new realities, will involve periods of distress as well as new personal growth, starting at diagnosis and extending for a lifetime.

As part of the process, it is common and normal for patients to experience periods of heightened distress. While these are often described loosely as 'anxiety' or 'low mood', it is important

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

not to unduly medicalise emotional reactions. Equally, it does mean that a 'universal' base level of psychosocial support is required for all patients. Within this, medical professionals hold a very important role in providing:

- compassionate and well-paced communication, that takes into account the person's state of mind and existing health beliefs when providing information.
- a reliable doctor–patient relationship, that respects the universal human need for 'safe attachment' to care and protection when vulnerable.
- active attention to the person's emotional responses and psychosocial context, and encouragement to engage with the necessary support resources.

As with any other clinical skill, these require explicit and active development, ideally through feedback and regular updates. Systematic screening for patient distress at key points, by using validated patient-reported measures (e.g. the Distress Thermometer and Concerns Checklist) is typically recommended. This can inform a 'supportive care plan' created collaboratively with the patient, which can encourage use of self-management resources (e.g. using a mobile app for guided stress-relief relaxation exercises, managing fatigue or improving sleep hygiene) and peer support resources (e.g. an online community or a creative group activity).

Where there is evidence of heightened distress and difficulties coping, an 'enhanced' level of support will be required to sustain the person through treatment and recovery. This involves active keyworking (e.g. by a nurse specialist or social worker) with more detailed exploration of psychosocial factors and needs (e.g. social support, work and financial stability, personal strengths and vulnerabilities) and provision of first-line psychological support (e.g. guidance to apply worry management coping skills).

Indications of pre-existing or newly symptomatic mental health difficulties (e.g. adjustment, mood or anxiety disorders, post-traumatic stress) or neurodevelopmental differences (e.g. autism, learning difficulties) require clinical assessment at a specialist level (e.g. clinical psychologists or liaison psychiatrists in psycho-oncology). The focus of intervention will be on the interactions between these difficulties and leukaemia treatment and recovery, and thus on-site participation in the haematology MDT is a requisite.

The compassionate and thoughtful care of people with leukaemia needs to be underpinned by equal attention to the wellbeing of healthcare staff. Combined with high workload demands, the long-term emotional labour, exposure to suffering and loss, and risk of moral injury, can and do take their toll. The impact is not only on the individual (burnout and mental health disorders) but on teamwork and the organisational levels of culture, safety and efficiency. Processes to protect and promote the resilience of staff, such as described in the ABC framework (West and Coia 2019) need to be a central concern for medical leadership and intrinsic to modern healthcare environments.

Social support is a critical, yet under-utilised resource when undergoing cancer care. Under-utilisation occurs in two conditions: (i) when patients fail to seek out information, material assistance and emotional support from family and friends or (ii) when family and friends fail to meet the individualised needs and preferences of patients. Patient's supportive groups run by departments, hospitals, organisations or charities might provide major social support to patients and relatives.

Most cancer patients experience financial problems. This might be more relevant to patients with acute leukaemia, as profound immunosuppression and delayed immune reconstitution may result in prolonged absence from work with devastating financial losses. Therefore, governmental bodies, health boards, organisations and charities must do more to ensure people living with cancer are signposted to good-quality specialist and timely welfare benefits support to help them cope and manage the financial impact of their diagnosis.

Reproductive aspects

Fertility preservation is an emerging discipline. As survivorship increases, quality of life issues such as the ability to have a child become increasingly important. Assisted reproductive technologies are now available that are effective and rapidly available to patients facing potentially sterilising treatment.

Options for males

Cryopreservation of sperm is a technique that has been available for decades for men and post-pubertal boys. Semen is produced by masturbation, and now with the use of intra-cytoplasmic sperm injection, treatment is effective even when only very small concentrations of sperm are available. In prepubertal males, there have been attempts to surgically recover spermatogonial cells directly from the testes for subsequent autotransplantation. Although promising, this remains experimental (Gul *et al.* 2020).

Options for females

The mainstay of fertility preservation for post-pubertal females is egg cryopreservation (or embryo-cryopreservation for women with male partners). Rapid treatments starting at any time of the menstrual cycle using gonadotrophin-releasing hormone (GnRH) antagonists to control the pituitary gland and follicle-stimulating hormone stimulation to produce multiple eggs followed by egg collection and freezing allow treatments to

occur within 12 days. In certain situations, this short delay prior to chemotherapy or radiotherapy may be acceptable to clinicians and patients. Pregnancy results using frozen eggs and embryos are now nearly equivalent to those with fresh embryos (Human Fertilization and Embryology Authority 2016). These results remain constant and do not reduce as the patient ages. Although not ideal, there is some limited experience with patients having ovarian stimulation and egg collection post-chemotherapy. In many cases despite these advances, there remain concerns around any delay prior to oncology intervention. In these patients, laparoscopic ovarian cortex collection can be performed as an urgent day-case procedure. The ovarian cortical biopsies contain many hundreds of immature eggs. This tissue can then be cryopreserved with a view to either future auto-transplantation, or *in vitro* maturation to create embryos for transfer. There have now been several hundred pregnancies following auto-transplantation (both spontaneously and following *in vitro* fertilisation), but access to this approach remains limited (Centerport *et al.* 2019). It has also been used successfully in prepubertal girls. Careful analysis of transplanted tissue is critical to avoid transferring cancerous cells back into a cured patient.

The use of GnRH agonist treatment as an ovarian protectant during cryopreservation is widespread but the evidence to support this remains somewhat equivocal. For those patients who have no time to undergo fertility preservation of their own gametes, treatments utilising egg and sperm donation remain effective. In women who have had pelvic radiotherapy or hysterectomy, surrogacy can be considered. For many patients, the risk of sterility following cancer treatment can be difficult to quantify. Dealing with this uncertainty remains an important part of the fertility preservation consultation. Guiding principles of fertility preservation include counselling, rapid access to fertility clinics and if appropriate treatment, individualised advice and honest and realistic management of expectation.

Blood count abnormalities

Anaemia

Chemotherapeutic agents induce anaemia through directly impairing haemopoiesis, including synthesis of red blood cell (RBC) precursors, in the bone marrow. Nephrotoxic effects of particular cytotoxic agents (e.g. platinum-containing agents) may lead to anaemia through decreased renal production of erythropoietin. The malignancy itself can lead to or exacerbate anaemia through bone marrow infiltration or production of cytokines that lead to iron sequestration, which decreases RBC production and may even shorten their survival. Additional potential contributors include nutritional deficiencies and changes in coagulation capability.

There is no good evidence to support a particular red cell transfusion policy in leukaemia. Therefore, the decision to conduct RBC transfusion should not be made strictly based on whether the haemoglobin level has reached a certain threshold or 'trigger'. As a general rule, transfusion is rarely indicated when the Hb level is greater than 90 g/L. However, different transfusion thresholds should be adopted, having taken into consideration three general categories of patients: (i) asymptomatic without significant comorbidities, for which observation and periodic reevaluation are appropriate, (ii) asymptomatic with comorbidities or high risk, for which transfusion should be considered and (iii) symptomatic, for which patients should receive transfusion. Older age and the presence of pre-existing cardiovascular, pulmonary or cerebral vascular disease may compromise a patient's ability to tolerate anaemia.

Use of erythroid-stimulating agents (ESA)

RBC production is normally controlled by erythropoietin, a cytokine produced in the kidneys. First introduced in 1989, ESAs are a synthetic, recombinant human erythropoietin that can stimulate erythropoiesis in patients with low RBC levels. The use of ESA remains very common in patients treated by chemotherapy for solid tumours and lymphoid malignancies. The American Society of Haematology (ASH) and the American Society of Clinical Oncology (ASCO) recommend a strict benefit/risk evaluation by clinicians for its use compared with RBC transfusions.

Concerns about ESA use come from some evidence of worsened outcomes in patients with solid malignancies observed firstly in randomised trials and later in three meta-analyses of randomised controlled trials. However, this association has been refuted by two other meta-analyses reporting no significant effect of ESAs on mortality or progression. In addition, several recent pharmacovigilance trials reported no decrease in survival with ESA use in patients with chemotherapy-related anaemia.

In patients receiving chemotherapy for acute myeloid leukaemia or undergoing allogeneic haemopoietic cell transplantation, the use of ESAs has not been clearly assessed.

Special considerations

All patients in whom allogeneic transplantation may be considered should receive cytomegalovirus (CMV)-negative or leucodepleted products until their CMV status is known. Patients treated who received purine analogue-based chemotherapeutic regimens (i.e. FLAG) should be supported with irradiated blood products. There is no good evidence to support a particular transfusion policy in leukaemia (Table 55.1).

Table 55.1 Summary of blood product transfusion in patients with acute leukaemia.

Packed red cell transfusion
- Transfuse if haemoglobin <70 g/L. However, consider underlying cardiovascular compromise and raise the threshold of transfusion as appropriate.
- Delay transfusion if features of hyperviscosity.
- Assess risk factors for transfusion associate circulatory overload (TACO): age, cardiac failure, renal impairment, fluid overload, hypoalbuminaemia, low body weight. Diuresis may be required if deemed at risk of TACO.
- Patients on purine analogue (e.g. fludarabine, cladribine) should receive irradiated blood components lifelong.
- Patients receiving allogeneic haematopoietic cell transplantation should continue to receive irradiated blood component until all four criteria are met:
  1 >6 months have elapsed since the transplant date
  2 the lymphocyte count is $>1.0 \times 10^9$/L
  3 the patient is free of active chronic graft versus host disease
  4 the patient is off all immunosuppression.

  If chronic GvHD is present or continued immunosuppressive treatment is required, irradiated blood components should be given indefinitely.
- All patients undergoing autologous haematopoietic cell transplantation should receive irradiated cellular blood components from initiation of conditioning chemo/radiotherapy until 3 months post-transplant (6 months if total body irradiation was used in conditioning).
- Patients with a history of Hodgkin Lymphoma are currently recommended in the UK to receive irradiated blood products lifelong.

Platelet transfusion
- Give prophylactic platelet transfusions to patients receiving intensive chemotherapy or undergoing allogenic haematopoietic cell transplantation to maintain a platelet count at or above 10 x 10^9/L.
- Increase threshold to 20×10^9/L in patients who are febrile and /or receiving antimicrobial therapy for suspected bacterial or fungal infection.
- Do not give platelet transfusions routinely for bone marrow aspirate and trephine biopsy, peripherally inserted central catheters, or traction removal of tunnelled central venous catheters.
- Patients requiring lumbar puncture, central-line insertion, percutaneous organ biopsy and for most invasive surgeries aim platelet count $>50 \times 10^9$/L.
- ABO-matched platelets should be used when available to maximise increments.
- Patients with HLA antibodies should receive HLA-selected platelet transfusion.

Plasma transfusion
- ABO identical plasma-derived product should be used as the first choice.

Thrombocytopenia

Prophylactic platelet transfusion should be administered to patients with thrombocytopenia resulting from impaired bone marrow function to reduce the risk of bleeding when the platelet count falls below a predefined threshold level (Table 55.1). This threshold level for transfusion varies according to the patient's diagnosis, clinical condition and treatment modality. The optimal platelet dose has not been identified.

Several recent clinical trials have addressed the question of which peripheral blood platelet count should trigger prophylactic transfusions, i.e. transfusions given to patients with severe thrombocytopenia without clinical haemorrhage. The prophylactic strategy seems beneficial, and available clinical guidelines generally recommend prophylactic transfusions when the peripheral blood platelet count falls to 10×10^9/L. This recommendation is supported by four randomised clinical trials that showed no statistically significant difference between the compared groups with regard to mortality, remission rates of the malignant disease, frequency of severe haemorrhages or erythrocyte transfusion requirements when comparing 10×10^9/L with a higher threshold. The use of 10×10^9/L as the threshold is in addition supported by a retrospective study comparing 224 patients transfused at a threshold of 20 and 256 patients transfused at a threshold of 10; these two groups showed similar bleeding incidence and erythrocyte transfusion requirements.

Another trial suggested that a policy of giving platelet transfusions only as treatment for bleeding might become a new standard of care in selected patients. However, higher rates of grade 3 or 4 bleeding events were reported amongst patients with acute myeloid leukaemia who received no prophylaxis, as compared with those who did receive prophylaxis. On the basis of these findings, and the results of a study by Stanworth *et al.*, the use of prophylactic platelet transfusions appears to be important as the proportion of patients

who had bleeding events of WHO grade 2, 3, or 4 was reduced by 7% overall in the group that received prophylactic platelet transfusions.

Platelet preparations

Platelets for transfusion can be prepared either by separation of units of platelet concentrates from whole blood, which are pooled before administration, or by apheresis from single donors. Comparative studies have shown that the post-transfusion increments, haemostatic benefit and side-effects are similar with either product. Thus, in routine circumstances, they can be used interchangeably. In most centres, pooled platelet concentrates are less costly. Single-donor platelets from selected donors are preferred when histocompatible platelet transfusions are needed. Both preparations can be stored for up to 5 days after collection at 20–24 °C with good maintenance of platelet viability.

Thrombopoietin receptor agonists

Thrombopoietin mimetics, such as romiplostim and eltrombopag, have been developed to treat patients with refractory immune thrombocytopenia. Thrombopoietin is a potent endogenous cytokine that binds to its receptor (c-Mpl) on megakaryocyte progenitors and stimulates a number of signal transduction events promoting megakaryocyte proliferation and maturation, and platelet release. Recent studies have suggested that use of thrombopoietin mimetics can be of clinical value in the treatment of refractory severe aplastic anaemia. Multiple studies have been undertaken to explore the potential utility of eltrombopag in the treatment of thrombocytopenia in patients with AML or MDS. Given the platelet-increasing effects, the *in vitro* and *in vivo* antileukaemia effects and the potential to chelate excess iron in multiply transfused patients, the utility of eltrombopag in patients with AML and MDS is actively being explored as a dual thrombopoietic and antineoplastic agent in different types of cancer.

Neutropenia

Granulocyte transfusions

Small randomised controlled studies over the past 20 years have shown some efficacy in the use of granulocytes; however, most of the studies lack power and provide inconclusive evidence. The largest study so far (RING study) showed no benefit of granulocyte infusions in patients post chemotherapy and post haemopoietic cell transplantation however, the analysis suggested that patients receiving a high dose (more than 0.6×10^9/L) might benefit. As HLA alloimmunisation has been reported in 17% of patients, patients who are transplantation candidates should be re-tested after granulocyte transfusions to ensure the absence of HLA antibodies directed against the potential donor. This might be more relevant in recipients of haploidentical transplants.

Granulocyte colony-stimulating factor

Myelosuppression due to cytotoxic drugs is a major limiting factor in the treatment of leukaemia. Neutropenia places patients at a high risk of infections, septicaemia and ultimately death. Three prophylactic strategies that have garnered much attention are: prophylactic antibiotics with antibacterial and antifungal agents, prophylactic Granulocyte colony-stimulating factor (G-CSF) and hospitalisation during severe neutropenia.

Recombinant human G-CSF (filgrastim) was licensed in 1991 by the United States Food and Drug Administration (FDA) for use in patients undergoing cytotoxic treatment for decreasing the incidence of febrile neutropenia and reducing the duration of neutropenia and fever following myeloablative chemotherapy. Products with similar activity (filgrastim biosimilars) are now available. The efficacy of G-CSF administered during and/or after induction chemotherapy in patients with acute leukaemia has been examined in randomised, multicentre trials producing conflicting results. Results of a meta-analysis suggest that use of colony-stimulating factors in febrile neutropenia accelerates neutrophil recovery and reduces the duration of hospitalisation. Practical use of G-CSF was limited by relatively short duration of its effects (half-life 3–4 hours, often requiring daily administration. Attachment of polyethylene glycol (PEG) molecule to filgrastim extended its half-life allowing administration every 2 weeks. As G-CSF overstimulates myeloid precursors, it changes appearance of diagnostic bone marrow specimens which may sometimes complicate their interpretation.

Hyperleukocytosis and leukostasis

Hyperleukocytosis is usually defined as a white blood cell count over 100×10^9/L. Whereas in chronic lymphocytic leukaemia (CML), this is rarely symptomatic, in chronic myeloid malignancies, and particularly in acute leukaemia (both myeloid and lymphoblastic) hyperleukocytosis is associated with a number of clinical problems. These include organ failure and early death secondary to leukostasis, bleeding, sometimes due to disseminated intravascular coagulation, and tumour lysis syndrome (TLS). Many laboratory tests and some clinical tests became unreliable in presence of hyperleukocytosis.

Although hyperleukocytosis leads to significant increase in leukocrit (fractional volume of leucocytes), it does not normally lead to increased blood viscosity due to simultaneous decrease of haematocrit associated with bone marrow infiltration. However, blood passage through microcirculation is impaired, and this may be related to the cell size or more likely to the interactions between leukaemic cells and the endothelium, mediated by adhesion molecules. Clinical symptoms and some laboratory phenomena associated with hyperleukocytosis may therefore arise in patient with white blood cell counts as low as 50×10^9/L.

In CML, patients usually remain asymptomatic unless the white blood cell count exceeds 200×10^9/L and even then, symptoms are rare or subtle. Headaches and retinal haemorrhage are

relatively common, sometimes causing impaired vision. Priapism may occur in male patient. Pulmonary leukostasis is extremely rare in CML, even with very high white blood cell counts (above 500×10^9/L). On the other hand, in patients with acute leukaemia, particularly in acute monoblastic leukaemia, pulmonary manifestation (tachypnoea, dyspnoea and cough) may appear early and be the first presentation of leukaemia. Chest radiographs may show diffuse alveolar consolidation compatible with alveolar oedema, diffuse reticulonodular infiltrates compatible with the diagnosis of pneumonia or they can show normal chest findings. Renal leukostatis may lead to renal failure. Central nervous system manifestations reported in hyperleukocytic patients include visual blurring, dizziness, tinnitus, slurred speech, ataxia, confusion, somnolence and coma. Administration of diuretics for incorrect presumption of heart failure may worsen the symptoms. Patients with hyperleukocytosis are in a risk of brain haemorrhage. Respiratory failure and cerebral haemorrhage are the most common causes of death in these patients. In some patients, the hyperleukocytosis syndrome, particularly its pulmonary manifestations, does not develop until intensive chemotherapy is administered.

Management of patients is further complicated by a number of spurious or potentially spurious laboratory results. Measurement of arterial partial oxygen pressure is very unreliable as there is very rapid consumption of oxygen *in vitro* and even cooling of the blood sample on ice does not prevent a spuriously low result. Metabolic activity and fragility of blasts also cause spurious hyperkalemia and hypophosphataemia. Measured lactate levels are likely to be higher than *in vivo* and glucose levels lower. There is likely to be an overestimation of the haemoglobin concentration and of haematocrit. Platelet counts may be inaccurate.

Decisions about modality, intensity and urgency of treatment depend mainly on clinical symptoms and signs and the cytological type of leukaemia. There is no threshold absolute white blood cell count for the initiation of therapy. Supportive care with adequate hydration, oxygenation, correction of coagulation and administration of platelets is paramount. Increasing platelet transfusion threshold to 20 or 50×10^9/L seems appropriate. Leukapheresis should be considered if available as an initial treatment modality as it rapidly reduces signs and symptoms of hyperleukocytosis and reduces tumour mass, thus reducing the risk of TLS. As the mechanical removal of leucocytes have only a temporary effect, it should be followed by or coincide administration of chemotherapy. High doses of oral hydroxycarbamide may be preferred to urgent intensive intravenous chemotherapy as the latter is more likely to exacerbate symptoms of leukostasis and trigger TLS. In a retrospective study of 779 patients with AML (Stahl *et al.* 2020) who presented with a white blood cell count above 50×10^9/L clinical leukostasis was reported in 27%, and leukapheresis was used in 15%. Use of leukapheresis did not significantly impact 30-day mortality, achievement of remission or survival in multivariate analysis. Thirty-day mortality was 17%, median survival was 12.6 months amongst all patients, and 4.5 months amongst those ≥65 years. This very poor prognosis in older patients poses an ethical dilemma regarding appropriateness of intensive treatment in this clinical situation, especially as this often cannot be fully discussed with rapidly deteriorating patients.

Skin, nail, dental, auditory and visual complications

Skin

Skin reactions to drug therapy are extremely common. Chemotherapy drugs can cause dry skin, hyperpigmentation, discolouration, serpentine hyperpigmentation, photoallergic reactions and acne (mainly treatment with steroids). Radiation treatment can cause dry or red skin in the area being treated. Certain areas are more sensitive than others. Patients should be encouraged to use moisturising creams on a regular basis. They should protect their skin from the sun by wearing a hat and clothing that covers the arms and legs, and apply sunscreen with a sun protection factor of 30 or higher before going outside.

Nail changes include:

- Beau's lines (horizontal depressions of the nail plate). These skin reactions can occur a few weeks after a course of chemotherapy.
- Mee's lines (white horizontal discolouration of the nail plate involving the entire nail width).
- Leukonychia (white horizontal discolouration involving partial nail width).
- Onycholysis (separation or loosening of a fingernail or toenail from its nail bed).
- Onychodystrophy (malformation of the nail).

Nails should be trimmed and kept clean and gloves should be worn for house-cleaning. Occasionally antibiotics and antifungal agents might be necessary to treat underlying infections.

Radiation recall is a skin reaction that can occur when certain chemotherapy drugs are given after radiation therapy. It usually appears in the area of skin where the radiation was given. The skin becomes red and tender. It may peel or blister, resembling sunburn. Radiation recall may be treated with corticosteroids to lessen inflammation or chemotherapy may be delayed until the skin heals.

Dental problems

Dental care is very important. Leukaemia and chemotherapy can make the mouth sensitive, easily infected and likely to bleed. Patients should receive a complete dental examination and, if possible, undergo necessary dental care before chemotherapy begins, since any existing lesions that might normally lie dormant can flare up and become life-threatening once the patient is immunosuppressed. Dental extractions should preferably be

performed at least 10–14 days before the commencement of chemotherapy so that epithelisation of the extraction site has been completed prior to the initiation of treatment. Dental procedures that could trigger a bleeding episode or/and bacteraemia during the aplastic phase should be avoided. The use of saline or alcohol-free chlorhexidine mouthwash is very important, primarily during the phase of aplasia. Aesthetic restorations of the enamel defects are often necessary, especially after completion of treatment.

Auditory toxicity

The auditory system is vulnerable to many chemical exposures. Antibiotics, such as the aminoglycosides, and chemotherapeutic agents, such as cisplatin, can cause considerable ototoxicity in as many as 40% of patients. Dosage reduction and regular assessment with auditory studies during the course of therapy might reduce toxicity and prevent further damage.

Visual toxicity

Ophthalmic complications induced by cytotoxic chemotherapy are often underestimated and underreported due to priority given to other life-threatening conditions; however, most of the ophthalmic complications are reversible if detected early enough. Amongst the functional complaints are blurred vision, decreased colour vision, diminished visual acuity, diplopia, night blindness and photophobia.

Amongst the drugs used on a regular basis to treat leukaemia, cytarabine can cause conjunctivitis and methotrexate can cause oedema, ocular pain, blurred vision, photophobia, conjunctivitis and blepharitis. Chlorambucil has been reported to cause keratitis and daunorubicin excessive lacrimation and conjunctivitis. Alemtuzumab can cause optic neuropathy and endophthalmitis. Finally, imatinib can cause periorbital oedema in up to 47% of patients.

The possible reversal of some of these side-effects, if discovered in time, emphasises the need for clinicians to be aware of these ocular reactions and suggest immediate consultation by an ophthalmologist.

Infections

Infection in leukaemic patients may be life-threatening and significantly contributes to mortality and morbidity in both community and hospital settings. Patients with leukaemia are at risk for infections both due to severe and prolonged neutropenia and as a consequence of chemotherapy with impaired innate immunity and destruction to physical barriers. Despite advances in antimicrobial prophylaxis and treatment, as well as diagnostic methods such as fungal serology, leukaemia patients remain at risk for life-threatening infection which leads to increased mortality, morbidity, prolonged hospitalisation and higher treatment cost.

Patients with leukaemia are predisposed to infections with many organisms including normal commensals from the gastrointestinal tract, oropharynx or skin, but also numerous opportunistic pathogens, including those in the environment and nosocomial infections. Table 55.2 lists important pathogens in this patient cohort, and Table 55.3 lists the locations of access for endogenous bacteria frequently causing invasive infection.

Following the introduction of chemotherapy for the treatment of leukaemia, Gram-negative organisms were the leading cause of blood stream infections in neutropenic patients. There has been a shift in the aetiology of infections in the setting of leukaemia over more recent years with an increase in Gram-positive bacteraemias and the emergence of resistant Gram-negative organisms. This in part may be related to use of central venous catheters as well as the increasing use of quinolone antibiotic prophylaxis in this patient group. Patients with leukaemia are frequently exposed to antibiotics and the hospital environment, predisposing them to the risk of colonisation and infection with antibiotic-resistant strains.

Over the past decade, there has been a rise in infections with resistant-strains such as extended-spectrum beta-lactamase (ESBL)-production in Enterobacteriaceae (including carbapenemase-producing organisms), multidrug-resistant *Pseudomonas* spp. and *Acinetobacter* spp., vancomycin-resistant enterococcus (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). Table 55.3 lists those host factors responsible for increasing the risk and severity of infection. Most patients will have more than one such risk factor.

The risk of infection increases with the duration and severity of neutropenia. Bacterial infections can occur at any stage during neutropenia; however, fungal infections typically occur later with invasive *Candida* spp. infections after 7 days of neutropenia and invasive moulds such as *Aspergillus* spp. after around 14 days.

Invasive fungal infection (IFI) carries significant morbidity and mortality. An increased risk of IFI is associated with induction/re-induction chemotherapy for AML and MDS, the use of purine analogues (such as fludarabine, clofarabine), and in allogeneic stem cell transplant recipients with graft-versus-host disease requiring corticosteroids and prolonged immunosuppression including the use of tumour necrosis factor-α blockade (TNF-α). Patients classified as being 'high risk' should receive antifungal prophylaxis with agents active against both yeasts and moulds.

Neutropenic fever

Patients with neutropenic fever must undergo a thorough clinical assessment and receive prompt empiric antibiotic therapy according to institutional neutropenic sepsis guidelines. A comprehensive clinical examination should include the skin, cardiorespiratory system, abdomen, oropharynx, central venous catheter and perianal region.

Table 55.2 Important pathogens in leukaemia patients.

	Bacteria	Endogenous/exogenous source of infection, notable characteristics
Gram-positive bacteria	*Coagulase-negative staphylococci*	Endogenous
	Staphylococcus aureus	Endogenous
	Enterococcus spp.	Endogenous
	Viridans group streptococci	Endogenous
	Streptococcus pneumoniae	Endogenous
	Streptococcus pyogenes	Endogenous
Gram-negative bacteria	*Escherichia coli*	Endogenous
	Klebsiella spp.	Endogenous
	Enterobacter cloacae	Endogenous
	Pseudomonas aeruginosa	Endogenous or exogenous; associated with water; colonises medical devices
	Acinetobacter spp.	Exogenous Typically acquired in the intensive care unit environment. Forms resistant biofilms on devices, often multi-resistant, and survives in the environment
	Stenotrophomonas maltophilia	Exogenous Low-virulence organism typically unable to overcome host barriers. Infection associated with carbapenem use, to which it is constitutively resistant
Yeasts and moulds	*Candida* spp.	Responsible for both superficial skin and mucosal infections and fungemia
	Aspergillus spp.	Causes rapidly progressive pneumonia Responsible for ca. 90% of invasive fungal infection in this cohort, of which >90% are pulmonary
	Pneumocystis jirovecii	Rare in this group; but not negligible
	Cryptococcus spp.	Rare cause of pneumonia and meningitis
	C. immitis, H. capsulatum, B. dermatitidis, P. brasiliensis, sporotrichosis, P. marneffei	Endemic, geographically limited highly virulent pathogens
	Zygomycetes, Fusarium, Scedosporium spp.	Low pathogenicity; tendency to produce skin lesions, disseminated disease and high mortality; difficult to treat

Blood cultures must be taken at the onset of fever and prior to antibiotic administration. A minimum of two sets of blood culture is recommended, taken peripherally and from central venous catheters (from each lumen). Approximately 10–15 mL of blood should be inoculated in each bottle (aerobic and anaerobic) as volumes lesser or greater will reduce the diagnostic yield. Blood cultures are typically incubated for 5 days; most cultures from bacteraemic patients are positive within 24 hours. A full identification is usually obtained within 24–48 hours. Recent advances in molecular diagnostics now allow for the rapid identification of organisms.

Empiric antibiotic therapy

The majority of infections in febrile neutropenia are treated empirically as only 30% or less of cases have positive microbiological identification. Febrile neutropenia is a medical emergency with life-threatening complications. Mortality rates vary and can be as high as 40%. Gram-negative sepsis carries a mortality of over 95% if not recognised early and treated adequately. Therefore, febrile neutropenic patients must be immediately treated with broad-spectrum antibiotics with antipseudomonal activity, typically a β-lactam/β-lactamase inhibitor combination

Table 55.3 Locations of access for endogenous bacteria frequently causing invasive infection.

		Typical context of pathogenicity	Important considerations
Skin	*Coagulase-negative staphylococci*	Line-associated infections	Biofilm formers often highly antibiotic-resistant
	Corynebacterium spp.	Bacteraemia associated with indwelling devices	*C. jeikeium* has a predilection for bacteremia via iv devices in this group
	Propionibacterium acnes	Rare cause of endocarditis and septic arthritis	Invasive infection often associated with indwelling prostheses
Oropharynx	Anaerobes	Dental abscesses	Often associated with polymicrobial infections
	Viridans streptococci	Dental plaque and caries Opportunistic cause of infective endocarditis	Pre-existing gum and tooth disease may relapse during chemotherapy
Nasopharynx and upper respiratory tract	*Streptococcus pneumoniae*	Pneumonia, otitis media, bacteraemia, meningitis	Risk markedly increased in asplenia or decreased serum (IgG). Polyvalent vaccine available but not necessarily effective in leukaemia
GI tract	*Enterobacter cloacae*	Urinary tract infections, bacteraemia	Resistant to beta-lactam antibiotics except carbapenems in some countries
	Enterococcus faecalis	Bacteraemia, endocarditis, urinary tract infections	Typically replaced by *Enterococcus faecium* (which is more resistant) following antibiotic treatment
	Escherichia coli	Urinary tract infections, bacteraemia	Often spontaneously translocates from the gut in neutropenia
	Klebsiella pneumoniae	Urinary tract infections, bacteraemia	Often highly antibiotic-resistant. Forms resilient biofilms on plastic.

(e.g. piperacillin/tazobactam) or with a carbapenem (e.g. meropenem). The latter can also be used in most cases of penicillin allergy, although some cross-reaction does exist. The use of more than one empiric antibiotic of a different class may be employed to minimise the chance of treatment failure in view of the rising trend in antimicrobial resistance.

Haematology units must review their local and national bacterial epidemiology and resistance patterns at intervals and tailor the empirical therapy accordingly. Subsequent isolation of a pathogen and its sensitivities will then allow for evidence-based, focused de-escalation of antibiotic therapy in most cases based on clinical improvement. Current patterns of resistance in most countries suggest the addition of an aminoglycoside (e.g. gentamicin or amikacin) to the initial β-lactam/β-lactamase inhibitor combination or carbapenem.

In cases with MRSA colonisation or suspected central venous catheter infection, the use of additional therapy with vancomycin or teicoplanin is essential. Previous infections with resistant organisms should guide the choice of empiric antibiotics and this is particularly important at the present time with the global increase in Gram-negative resistance, including against carbapenems. Some countries (such as the UK) recommend testing for multidrug-resistant organism colonisation, usually by means of a pre-emptive rectal swab.

In patients presenting with febrile neutropenia, antibiotic treatment with a β-lactam/β-lactamase inhibitor combination (e.g. piperacillin/tazobactam) or carbapenem (e.g. meropenem), aminoglycoside (e.g. gentamicin or amikacin) and additional vancomycin or teicoplanin in the presence of a central venous catheter is recommended. Patients who are on long-term immunosuppression, such as those with graft-versus-host disease, may not mount a febrile response to infections and present with hypothermia ('cold sepsis'). These patients should be assessed as soon as they present and started on empiric antibiotics promptly as any delays may be fatal.

The first-line use of empiric antifungal treatment is generally not recommended. Antifungal treatment should be considered in neutropenic patients with unremitting fevers not responsive to broad-spectrum antibiotic therapy or in the presence of clinical or radiological signs supporting the diagnosis of an IFI.

The choice of antifungal treatment will depend on the evidence of pre-existing fungal colonisation (such as *Candida* spp. colonisation of skin/oropharynx), previous fungal isolates and local epidemiological surveillance of prevalent fungal pathogens.

Investigating neutropenic fever

Although most episodes of neutropenic fever are not associated with localising symptoms or signs, a thorough history and clinical examination are essential as this will then guide appropriate investigations to identify and treat the source of infection promptly.

Respiratory infections

A chest radiograph must be performed on all patients with respiratory symptoms at the time of presentation. The differential diagnosis includes bacterial, fungal and viral causes.

Aspergillus spp. are responsible for the majority of invasive pulmonary fungal infections in neutropenic patients and may be associated with cough, pleuritic pain and haemoptysis. Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) and the subsequent disease, coronavirus infectious disease-2019 (COVID-19) should be excluded in all patients in the current pandemic. Correct technique in performing nasopharyngeal/oropharyngeal viral swabs is essential and samples should be sent for real-time reverse transcription-polymerase chain reaction (RT-PCR) determined diagnosis of SARS-CoV-2, influenza, parainfluenza subtypes, adenovirus, metapneumovirus and respiratory syncytial virus.

Patients with prolonged neutropenia and persistent fevers despite broad-spectrum antibiotics should proceed to computed tomography (CT) imaging to assess for pulmonary +/− sinus fungal infection. High-resolution CT imaging is the preferred imaging modality and the presence of lung nodules or infiltrates with a halo sign in a neutropenic patient is highly indicative of invasive aspergillosis. The air crescent sign seen on high-resolution CT is often a late and non-specific finding. Radiological findings in patients with pulmonary mucormycosis include consolidation, mass-like lesions, lung nodules and cavitation. The presence of multiple nodules (>10 nodules) as well as pleural effusions appear to be more common in pulmonary mucormycosis than in invasive aspergillosis. The reverse halo sign is often described in pulmonary mucormycosis and is more specific for mucormycosis than invasive aspergillosis; however, the differential diagnosis may include infections such as pulmonary tuberculosis. The presence of diffuse and bilateral parenchymal abnormalities often suggests a diagnosis of either viral-induced pneumonitis or *Pneumocystis jirovecii* pneumonia. Bacterial infections due to *Pneumococcus* or *Haemophilus* spp. typically appear as more confluent areas of anatomically well-defined consolidation.

High-resolution CT has an important role in the diagnosis and management of COVID-19 pneumonia. The common CT findings among patients with COVID-19 pneumonia include predominantly peripheral, bilateral ground-glass opacities with or without consolidation and interlobular septal thickening producing a 'crazy-paving' appearance. Although an important diagnostic tool with high sensitivity for COVID-19 pneumonia, CT findings of COVID-19 pneumonia can be non-specific and may resemble various infectious and non-infectious complications often seen in the neutropenic/immunosuppressed haematology patient. Therefore, awareness and knowledge of radiological findings are important in the early diagnosis and treatment of infections in the haematology patient during the current pandemic. The duration of symptomatology, degree and duration of neutropenia or immunosuppresssion, ancillary diagnostic tests, and follow-up imaging is helpful in the differential diagnosis in the haematology patient presenting with fever and respiratory symptoms.

Broncheoalveolar lavage (BAL) +/− tissue biopsy in the appropriate clinical setting are preferred over radiological findings alone. BAL is a useful tool and can be safely performed, but in patients who have been on prolonged antimicrobials the diagnostic yield is reduced to approximately 20%. Bronchoscopy and BAL are recommended in patients with lung infiltrates on imaging with no clear cause for infection. BAL results (microscopy, culture/sensitivity and PCR) can help identify microorganisms and allow de-escalation of antimicrobials.

Galactomannan, which is a polysaccharide component of the *Aspergillus* spp. cell wall (as well as other closely related filamentous fungi), can be released by fungal hyphae into the blood and other body fluids during invasive growth. The presence of this antigen can be sustained for up to 8 weeks and the level of circulating serum or plasma galactomannan is indicative of fungal burden. However, there are some limitations; its sensitivity in diagnosing invasive aspergillosis is reduced by exposure to mould-active antifungals, and therefore absent galactomannan from serum or plasma in patients receiving mould-active antifungals should be interpreted with caution in parallel with clinical and radiological findings. Exposure to certain antibiotics (piperacillin/tazobactam, amoxicillin/clavulanic acid, ampicillin and phenoxymethylpenicillin) and diet can lead to false-positive results. BAL galactomannan is, however, more sensitive for detection of invasive pulmonary aspergillosis compared to serum in at-risk patients.

(1,3)-Beta-D glucan testing is not mould-specific, and is found in a wide range of fungal pathogens, for example *Candida* spp., *Aspergillus* spp., and *Pneumocystis jirovecii*, but is useful as an alternative serological diagnostic test especially for exclusion in the appropriate clinical setting. *Aspergillus* PCR on blood and BAL fluid also provides an additional diagnostic tool for the diagnosis of *Aspergillus* infection.

Other pathogens capable of causing pulmonary disease, such as *Nocardia* spp., *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Histoplasma* and *Cryptococcus* spp., as well as rare moulds should be part of the differential diagnoses in this highly

vulnerable group. Patients with leukaemia are at risk of *Mycobacterium tuberculosis* infection. The tuberculin skin test and interferon-γ release assays are not reliable diagnostic tools in this patient group as the tests require intact cellular immunity. Detection of acid-fast bacilli under microscopy and/or culture or PCR positivity for *M. tuberculosis* should prompt combination therapy under expert advice and relevant infection control measures.

Other opportunistic respiratory pathogens such as *Pneumocystis jirovecii*, *Toxoplasma gondii*, atypical mycobacteria and *Nocardia* spp. should be considered in patients who do not respond to conventional antibiotics and antifungals. Patients with *Pneumocystis jirovecii* pneumonia typically present with dry cough, dyspnoea and hypoxia (especially on exertion). Chest radiography is usually the initial imaging modality but findings are non-specific and up to one-third of infected patients may have normal radiographic findings. The hallmark finding of *Pneumocystis jirovecii* pneumonia on high-resolution CT is bilateral ground-glass opacities and less frequently, consolidation, nodules, unilateral infiltrates, pleural effusions, cysts and spontaneous pneumothorax. The detection of *Pneumocystis jirovecii* DNA by quantitative real-time PCR on BAL fluid or sputum specimens is suggestive of active infection (or colonisation if low levels are detected). Elevated serum beta-D glucan levels can be used to support the diagnosis of probable *P. jirovecii* disease in the appropriate clinical context provided other IFIs have been excluded.

The range of possible respiratory pathogens highlights the importance of obtaining diagnostic sputum, BAL and/or tissue samples and PCR-based diagnostics whenever possible. Potential delays in diagnostic procedures may lead to clinical deterioration and missed opportunities in obtaining diagnostic yield, especially in patients on prolonged antimicrobial therapy.

Gastrointestinal infections

Patients with leukaemia commonly suffer from gastrointestinal symptoms which may be chemotherapy-induced or infective in origin. Chemotherapy toxicity leads to mucosal ulceration and defects throughout the gastrointestinal tract. Symptoms such as diarrhoea, abdominal pain and fever can be caused by either chemotherapy-induced destruction of gut mucosa (with consequences for bacterial penetration to deeper tissues and translocation to the blood), or by a primary, local process of invasion. Neutropenic enterocolitis/typhilitis is a life-threatening condition with a high mortality rate. Early recognition and prompt treatment are essential. Most patients respond to broad-spectrum parenteral antimicrobials, typically a carbapenem (with its anaerobic activity) alongside supportive management with fluids, electrolyte replacement and analgesia. Anti-diarrhoeal agents should be avoided.

Neutropenic patients with persistent fever despite antimicrobial treatment and without evidence of an infective focus should have abdominal imaging. This should be in the form of an ultrasound scan in the first instance to investigate for evidence of hepatosplenic candidiasis. Any identified lesions in the liver should be biopsied if at all possible. Whilst *Candida* spp. remains the most likely causative pathogen of multiple hepatosplenic lesions, a very broad range of alternative pathogens exists including many species of bacteria, mycobacteria and fungi.

Patients with upper gastrointestinal symptoms such as epigastric pain and intractable nausea/vomiting should undergo endoscopy with biopsies to exclude infection with *Candida* spp., *Cytomegalovirus* or *Herpes* spp.-related oesophagitis/gastritis.

Severity of *Clostridium difficile* infection ranges from benign diarrhoea to lethal pseudomembranous colitis. *Clostridium difficile* should always be excluded with relevant stool antigen and toxin tests as well as abdominal CT in cases with severe symptoms. Treatments include vancomycin, metronidazole and fidaxomicin. Intestinal microbiota transplant can be considered in patients with multiple recurrences or refractory *Clostridium difficile* infection despite antibiotic treatment. Neutropenic patients with perianal pain should undergo MRI to further investigate the cause of the pain. Abnormalities such as fistulas and haemorrhoids can lead to bacterial infection and require treatment with broad-spectrum antibiotic in order to prevent the spread of infection.

Skin infections

Skin lesions often represent metastatic spread of infection and punch biopsies should be performed for histopathology, viral PCR and culture for bacteria, fungi and mycobacteria. *Ecthyma gangrenosum* is a serious skin infection characterised by ulcerative lesions with necrotic centres. These lesions are typically seen in the perineum, axillae and submammary regions as well as limbs and trunk, and are caused by systemic *Pseudomonas aeruginosa* infection which is associated with a high mortality rate. These patients present with sepsis/shock with concurrent *Pseudomonas* bacteraemia. Fungi, particularly *Aspergillus* spp., *Candida* spp. and *Fusarium* spp., can also disseminate to the skin causing papular lesions with necrotic centres. The latter organisms also often involve the retina therefore fundoscopy is mandatory in these cases to prevent permanent blindness. Infection with atypical mycobacteria can present with skin lesions, typically nodules.

Infections due to central venous catheters

A central venous catheter is commonly used to deliver chemotherapy and is a common source of infection in the neutropenic patient. Central venous catheters are susceptible to colonisation with bacteria, fungi and mycobacteria. The presence of fever in the absence of an infective focus should always prompt the possibility that the device is responsible for the fever, and blood cultures should be taken via the line lumens (each being cultured separately) as well as peripherally. Central venous catheters should be removed promptly if there is

evidence of infection at the catheter site, or in the presence of fevers associated with line use.

Central venous catheters should be removed in the setting of bacteraemia with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella* spp., *Stenotrophomonas* spp. and *Candida* spp. due to their virulence and ability for haematogenous spread to heart valves and joints. These organisms tend to adhere to plastic and lead to biofilm formation, resulting in failure of antimicrobial therapy.

Intraluminal colonisation of the catheter (with no local signs of infection) can be treated with antibiotic line locks in an attempt to sterilise the line. However, infection around the catheter site will usually require catheter removal as the infection is at the avascular tissue/plastic interface and therefore not amenable to antibiotic-mediated killing.

In the context of a febrile neutropenic patient with a suspected line infection, vancomycin or teicoplanin should be added to the empirical antibiotic regimen. Lines infected with coagulase-negative Staphylococci can be salvaged with combined systemic antibiotics and antibiotic line locks. If the fevers do not resolve despite line removal, then an ultrasound scan should be performed to exclude an underlying infected thrombus.

Invasive fungal infection

IFIs are a major cause of morbidity and mortality in leukaemic patients. The risk of IFIs is highest for patients undergoing remission induction chemotherapy for acute myeloid leukaemia and myelodysplastic syndrome, those treated with purine analogues (fludarabine, clofarabine), prolonged duration of severe neutropenia, and recipients of allogeneic haematopoietic cell transplant (HCT) with graft-versus-host disease requiring steroids and multiple lines of immunosuppressive therapy. This group of high-risk patients should therefore receive primary antifungal prophylaxis with a mould-active antifungal agents. There is sufficient data to support posaconazole as the prophylactic agent of choice in this high-risk population.

Liposomal amphotericin, echinocandins and posaconazole/voriconazole form the mainstay of antifungal treatment. It may be noted, however, that the azoles may be associated with significant side-effects and have unpredictable metabolism and drug interactions, making their use challenging.

Liposomal amphotericin is active against almost all fungal pathogens, while voriconazole has a similar broad spectrum of action except for lacking activity against *Mucor* spp. Fluconazole has good activity against *C. albicans*, most non-albicans *Candida* and *Cryptococcus* spp., but is not active against moulds, for example *Aspergillus* spp., *Mucor* spp. and *Fusarium* spp. Flucytosine, the echinocandins and itraconazole have a similar spectrum of activity to fluconazole but itraconazole is active against *Aspergillus* spp., and the echinocandins lack activity against *Cryptococcus* spp.

The most virulent pathogen in this setting is *Aspergillus* spp. – most commonly fumigatus or flavus. These moulds are not susceptible to fluconazole. For this reason, the use of mould-active agents in high-risk patients for IFI is critical. The choice of prophylactic agent varies between centres and between countries, and the choice is dependent on local preference and cost-benefit analysis. Mould-active agents include the later generations of triazole antifungals, such as itraconazole, voriconazole (almost exclusively reserved for treatment rather than prophylaxis) and posaconazole. Isavuconazole, a novel triazole drug with broad-spectrum antifungal activity, has shown safety and efficacy in treating IFIs.

There is robust data showing the effectiveness of posaconazole in reducing the incidence of invasive mould infection in these high-risk patients; however, the drug is expensive and (like other azoles except fluconazole) has variable and unpredictable oral absorption profile in its syrup form. This relates to its lipophilic properties, shared with the other azoles apart from fluconazole. All rely on an acid pH for ionisation and absorption and this is often impeded by the concurrent administration of powerful antacid medication, including H2 blockers and proton pump inhibitors. A different oral tablet formulation of posaconazole with better absorption is now available.

Due to the high pharmacokinetic variability of azoles, regular therapeutic drug monitoring is essential to optimise dosage in order to maximise the therapeutic potential while limiting adverse reactions. Recent data as well as recommendations from the Infectious Diseases Society of America support therapeutic drug monitoring for the mould-active triazole antifungals such as itraconazole, voriconazole and posaconazole.

Antibiotic resistance

Antibiotic resistance is a major public health concern. The most important prevention is to ensure proper use of antibiotics as well as infection control measures to reduce transmission of these resistant strains in hospitalised patients. These include proper hand hygiene, contact precautions and regular environmental cleaning. Implementation of antibiotic stewardship should be encouraged to promote careful use of antimicrobials and local surveillance of antimicrobial resistance patterns.

Identification of the colonisation or infection in a patient with an antibiotic-resistant organism should immediately prompt review of the current treatment as well as consideration of the empiric antibiotic regimen to be used should they become febrile at a later date. Table 55.4 shows major antibiotic resistance mechanisms; of note is that several may coexist, rendering treatment choices exceedingly limited if not entirely impossible.

Investigation of persistent fever

Some patients continue to have persistent fever despite broad-spectrum antimicrobials. Frequent changes in antimicrobials

Table 55.4 Mechanisms of resistance and important considerations flowing therefrom.

Antibiotic	Bacteria	Comments
Chromosomal *AMP C* β-lactamase	*Citrobacter freundii* *Enterobacter* spp. *Morganella morganii* *Proteus* spp. *Serratia* spp.	Able to break down penicillins and cephalosporins. Treatment with third-generation cephalosporins (includes ceftazidime and ceftriaxone) induces hyperproduction of resistance enzymes. Not inhibited by clavulanic acid. Usually require treatment with a carbapenem.
Extended spectrum β-lactamases	Most commonly found in: *E. coli* *Klebsiella pneumoniae* Described in other Gram-negative bacilli	Confer resistance to penicillins and cephalosporins. Usually inhibited by a β-lactamase inhibitor, such as clavulanic acid.
Carbapenem	Enterobacteriaceae, particularly: *E. coli* *Klebsiella pneumonia* *Pseudomonas aeruginosa*	Multiple known mechanisms of acquired carbapenem resistance. Very challenging management. May remain sensitive to tigecycline, colistin, fosfomycin or aminoglycosides. These infections often require dual or triple therapy combinations.
Methicillin	*Staphylococcus aureus*	Resistant to all β-lactams due to altered penicillin-binding site. All UK isolates so far sensitive to glycopeptides (vancomycin and teicoplanin). Variable sensitivity to doxycycline, aminoglycosides, trimethoprim and rifampicin. Alternatives: daptomycin, linezolid.
Vancomycin	*Enterococcus* spp.	Vancomycin-resistant enterococci are typically acquired through exposure to glycopeptide treatment and can lead to persistent carriage in the GI tract

may not be necessary unless the patient is clinically deteriorating with rising inflammatory markers, haemodynamic instability or there is evidence of progressive infection either radiologically or clinically. Patients must be reviewed daily, and every effort should be made to diagnose the source of infection. Blood cultures should be taken regularly in the patient with persistent fever, and repeat high-resolution CT of chest, CT sinuses and abdominal imaging should be considered in the appropriate clinical setting. Consideration should also be given to removing the central venous catheter if no other infective source is identified.

Opportunistic infections such as tuberculosis, atypical mycobacterial infection, toxoplasmosis, and in patients with geographic risk factors, malaria, brucellosis and Strongyloides should be considered in patients with ongoing fevers who do not respond to conventional antibiotics and antifungals. Travel history, country of origin and pre-existing serology may be helpful to guide investigations and management in the setting of pyrexia of unknown origin.

When to stop anti-infective agents

Oral antibiotic step-down is generally not recommended once sepsis has resolved. Intravenous antibiotics can be stopped once there is clinical improvement (radiological improvement can sometimes lag) and upon resolution of fever. However, there are special situations, for example in the setting of *Staphylococcus aureus* blood stream infection, in which a prolonged course of treatment is indicated and after completion of at least 14 days of intravenous therapy an oral sequential therapy can be considered to complete the desired course of treatment. The decision regarding a prolonged course of antibiotics should be made following consultation with the Microbiology/Infectious Diseases team. The prolonged use of antibiotics without a clear indication should be discouraged as this practice promotes antibiotic resistance and predisposes to *C. difficile* infection.

Antifungal therapy, however, should continue in the setting of continued fevers with worsening radiological changes, prolonged neutropenia and ongoing steroid treatment/immunosuppression for graft-versus-host disease. IFIs should be treated for a minimum of 6 weeks; however, this is very much guided by patient factors such as the anticipated duration of neutropenia and ongoing immunosuppression, and some patients may need a very prolonged course of antifungal therapy. Antifungal stewardship and collaboration between the Haematology, Microbiology and Respiratory teams should be encouraged in order to rationalise antifungal usage, managing drug toxicity and the associated cost implications of these agents.

Special considerations in allogeneic haematopoietic cell transplant recipients

Infections are a common cause of morbidity and mortality following an allogeneic HCT and these patients are at risk of opportunistic infections for long periods of time, starting with the conditioning therapy in the peri-transplant period to potentially several years following the transplant.

Transplant conditioning regimens include the use of high-dose chemotherapy +/− radiotherapy which causes prolonged impairment of haematopoiesis rendering the recipient neutropenic with impaired phagocytic function. It also leads to destruction of mucocutaneous barriers, and consequently, the skin and gastrointestinal tract allow translocation of bacteria, fungi, and other organisms causing potentially serious infections. Herpes simplex virus (HSV) reactivation can occur during this period and it is recommended that all cell transplant recipients receive prophylactic antivirals from the start of conditioning. Following sustained neutrophil engraftment there is restoration of effective phagocytic function, which greatly reduces the risk for bacterial and fungal infections.

These patients rapidly lose all B- and T-lymphocytes following conditioning, along with immune memory from past exposure to infections and vaccination. Recipients of allogeneic HCT will continue to have impaired cell-mediated immunity, the extent of which is influenced by severity of graft-versus-host disease and degree of immunosuppression.

One of the most important infections in the allogeneic cell transplant context is CMV disease. Prior to the introduction of the first effective anti-CMV agent (ganciclovir) in the late-1980s CMV pneumonitis was one of the most common causes of post-transplant mortality, with significant morbidity arising from CMV enteritis and hepatitis. However, regular screening for CMV infection/reactivation following transplantation using whole blood or plasma RT-PCR has allowed the early detection and treatment to commence prior to the development of CMV disease, and due to this improvement in detection and the availability of effective antiviral agents CMV has become an uncommon cause of post-transplant mortality. Interestingly, CMV also potentiates infection with opportunistic pathogens, especially in patients with active graft-versus-host disease through complex interactions with the immune system. Treatment options for CMV infection include ganciclovir, valganciclovir, foscarnet and cidofovir. Letermovir is a novel antiviral agent that inhibits CMV replication and is now licensed for prophylaxis in CMV-seropositive allogeneic HCT recipients.

Allogeneic HCT recipients have immune system dysfunction for months to years following the transplant and have impaired cell-mediated and humoral immunity putting them at risk for CMV, varicella-zoster virus (VZV), EBV-related post-transplant lymphoproliferative disease, respiratory viruses, and infections with encapsulated bacteria (e.g. *Haemophilus influenzae* and *Streptococcus pneumoniae*).

Allogeneic HCT recipients should also routinely be re-vaccinated, starting at 6–12 months post-transplant (Table 55.5). However, there is no standardised vaccine schedule for HCT recipients and individual transplant centres should review available guidance in order to make recommendations.

There are now guidelines and recommendations for COVID-19 vaccination in patients with haematological malignancy and allogeneic HCT recipients. This is a new and evolving area and further recommendations will likely follow in due course. At the present time, the immunogenicity and efficacy of the SARS-CoV-2 vaccines in allogeneic HCT recipients and the impact of both graft-versus-host disease and prolonged immunosuppression on vaccine responses remains unknown.

Conclusions

The two major causes of outcome failure in the management of patients with leukaemia are disease progression/relapse and fatal infections. These patients develop infections both as a consequence of the disease itself and as a result of the therapy necessary for remission and/or cure. Febrile neutropenia and sepsis of bacterial origin represent a clinical emergency and the protocol of antibiotics used must be appropriate and sufficient. Recent global changes in Gram-negative resistance patterns make these choices increasingly difficult, and rectal screening for carriage of multidrug-resistant Gram-negative organisms ahead of the period of neutropenia is becoming more widespread and clinically important. Equally, the correct management of fungal infection relies on risk stratification and in appropriate cases, prophylaxis. The utility of fungal diagnostics is currently suboptimal making empiric antifungal treatment, usually based on CT scanning and exclusion of other causes of fever, the norm. Response rates and 'cure' in such patients often relates to the prognosis of the underlying leukaemia; persistent malignant disease very significantly impacts on the ability of these drugs to eradicate the fungal infection. Finally, viral infections remain a significant threat for those in whom cell-mediated immunity is poor, either subsequent to the disease or to chemotherapy. In addition, many such infections can rapidly spread through an inpatient haematology unit emphasising the need for strict infection control precautions.

Metabolic complications

Fluid balance

Fluid balance should be monitored carefully in patients treated with intensive chemotherapy or haematopoietic cell transplantation. Daily weight monitoring is equally important. Fluid input/output monitoring should take place several times within each 24-hour period in order to be able to observe unwanted

Table 55.5 ECIL recommendations for allo-HSCT recipients.

Vaccine	No. of doses*	Time post-HCT to initiate vaccine	Grading
Influenza (inactivated)	1 (or 2, special cases)**	>6 months As long as patient is judged to be IS Yearly, lifelong from 3 months in case of a community outbreak	AIIr BIIr BIIr
Measles–mumps–rubella			
• Measles	1 (2 in children) MMR	≥24 months	BIIu
In sero(-) patients, with no GVHD, no IS, no REL of underlying disease, and no IGIV at least 8 months		≥12 months in case of measles outbreak in patients with low grade IS	CIII
• Rubella	1 MMR	≥24 months	CIIu
In sero(-) women and of childbearing potential, with same precautions as for measles vaccine			
Virus hepatitis B***			
• Sero(-) patients before HCT and patients vaccinated pre-HCT but lost their immunity at 6 months)	3†	6–12 months 6-12 months	BIIt BIII
• Previously infected and anti-HBs <10 IU/L		Vaccine before transplant	BIII
• Sero(-) patients with a donor with positive anti-HBc			
Human papilloma virus (HPV) Follow recommendations for general population in each country	According to official label	From 6 to 12 months	BIIu
Inactivated polio	3‡	6–12 months	BIIu
Live-attenuated varicella vaccine	1	Can be considered in sero(-) patients, with ALL the following: >24 m from HST, no GVHD, no IS, no REL of the underlying disease, and no IGIV in the last 8 months	BIIr
	2	The addition of a second dose in adults may be considered in patients who were sero(-) before HST or had no history of VZ infect	
Live-attenuated zoster vaccine	Not recommended		DIII
Pneumococcal conjugate (PCV)	3	3 months	AI
Polysaccharidic vaccine in case of GVHD, use PCV instead of PPS for this 4th dose (BIIr)	1	12 months (no earlier than 8 weeks after last PCV)	BI
Meningococcal conjugate (in accordance with country recommendations and local prevalence)	2	From 6 months For men-C or tetravalent vaccine For men-B vaccine	BIIu BIII
Haemophilus influenzae conjugate	3	3 months or 6 months	BIIr
Diphtheria-tetanus (DT is preferred to Td)	3‡	From 6 months	BIu
Pertussis (acellular) (DTaP is preferred over Tdap)	3	From 6 to 12 months	CIII

Note for auto-HCT: same recommendations but grading changes for some vaccines: influenza BIIr (instead AII); PCV BIII (instead AI).
* If not specified otherwise, the interval between dose is 1 month.
** Influenza: A second dose of influenza vaccine, after 3–4 weeks from the first, may have a marginal benefit and should preferably be considered in patients with severe GVHD or low lymphocyte count (BIIr) and also for the patients vaccinated early (from 3 months after transplant) (BIIr). Children ≥6 months through 8 years, receiving influenza for the first time after transplant, should receive a second dose at least 4 weeks after the first dose.
*** HBV. After post-transplant vaccination, if anti-HBs is <10 mIU/mL, an additional three doses should be considered, but the benefit of this second series of vaccination is uncertain. IDSA guidelines (Rubin *et al.* 2014) give the same recommendations (strong, low). For adolescents and adults, a high dose of vaccine (40 µg) is recommended for these booster doses (strong, low).
† Three doses: interval 0, 1, and 6 months.
‡ At 1–2 months interval.
Source: Cordonnier *et al.* (2017)/ECIL.

changes in fluid balance and to be able to intervene, if necessary. Elderly patients are more susceptible to fluid imbalance due to underlying comorbidities, including congestive heart failure and renal insufficiency, and therefore should be monitored more closely.

Vomiting is common in patients on chemotherapy and in combination with diarrhoea and sweating due to pyrexia might rapidly lead to dehydration. Potential vasodilatation due to sepsis might exacerbate hypovolaemia and therefore strict monitoring and fluid replacement should be undertaken.

Hydration prior to cytotoxic agents

Hydration, urine alkalinisation (pH 7–8), and electrolyte monitoring can prevent hyperuricaemia, hyperphosphataemia, hypocalcaemia and hyperkalaemia. This is more relevant at diagnosis as the disease bulk is increased and the risk appears to be higher. The goal hydration is to improve renal perfusion and glomerular filtration and induce a high urine output to minimise the likelihood of uric acid or calcium phosphate precipitation in the tubules. However, intravenous hydration can lead to potentially dangerous fluid overload in patients with underlying acute kidney injury or cardiac dysfunction. Close monitoring is therefore important to prevent complications associated with TLS or fluid overload.

Tumour lysis syndrome

TLS is an oncologic emergency that is caused by massive tumour cell lysis with the release of large amounts of potassium, phosphate and nucleic acids into the systemic circulation. Catabolism of the nucleic acids to uric acid leads to hyperuricaemia, and the marked increase in uric acid excretion can result in the precipitation of uric acid in the renal tubules. It can induce renal vasoconstriction, decreasing renal blood flow and inflammation, resulting in acute kidney injury. Hyperphosphatemia with calcium phosphate deposition in the renal tubules can contribute to acute kidney injury. TLS is a frequent complication of advanced-stage Burkitt lymphoma and acute leukaemia with hyperleukocytosis. The symptoms associated with TLS reflect the associated metabolic abnormalities (hyperkalaemia, hyperphosphatemia and hypocalcaemia). These may include nausea, vomiting, anorexia, lethargy, cardiac dysrhythmias, seizures, muscle cramps, tetany, syncope and possible sudden death.

Aggressive intravenous hydration is the cornerstone of preventing TLS and is recommended prior to therapy in all patients at intermediate or high risk for TLS. Patients at risk for TLS should receive 2–3 L/m^2 per day of fluid. Urine output should be monitored closely and maintained within a range of 80–100 mL/m^2 per hour. Diuretics can be used to maintain the urine output, if necessary, but should not be required in patients with relatively normal renal and cardiac function. The best diuretic for patients with TLS is unknown; loop diuretics such as furosemide appear preferable because they not only induce diuresis, but may also increase potassium secretion.

Allopurinol is a hypoxanthine analogue that competitively inhibits xanthine oxidase, blocking the metabolism of hypoxanthine and xanthine to uric acid. Allopurinol decreases the formation of new uric acid and reduces the incidence of obstructive uropathy in patients at risk for TLS. Because it acts by decreasing uric acid formation, allopurinol cannot reduce the serum uric acid concentration already present before treatment is initiated. Thus, for patients with pre-existing hyperuricaemia (serum uric acid $\geq$7.5 mg/dL [446 µmol/L]), rasburicase is the preferred agent. Allopurinol increases serum levels of the purine precursors hypoxanthine and xanthine, which may lead to xanthinuria, deposition of xanthine crystals in the renal tubules, and acute kidney injury. Since allopurinol also reduces the degradation of other purines, dose reductions of 65–75% are needed in patients being treated with mercaptopurine or azathioprine. Allopurinol has the potential to interact with a number of other drugs, including cyclophosphamide, high-dose methotrexate, ampicillin and thiazide diuretics. Amongst patients who are unable to take oral medications, intravenous allopurinol can be administered. Treatment is normally initiated 24–48 hours before the start of induction chemotherapy and is continued for up to 3–7 days after treatment, until there is normalisation of serum uric acid and no other laboratory evidence of tumour lysis.

An alternative approach to allopurinol for lowering serum uric acid levels is to promote the degradation of uric acid by the administration of rasburicase. This is an active enzyme, urate oxidase (uricase), which catalyses oxidation of uric acid to the much more water-soluble compound allantoin. Rasburicase rapidly breaks down serum uric acid and is effective in preventing and treating hyperuricaemia and TLS. This rapid reduction in serum uric acid is in contrast to the effect of allopurinol. The enzymatic oxidation of uric acid results in an oxidative stress to the erythrocytes. Uric acid itself is a potent free-radical scavenger, so its rapid removal further exacerbates this oxidative stress. Administration of rasburicase therefore in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency therefore triggers haemolysis and methaemoglobinaemia. This may happen even in patients with mild G6PD deficiency, including females, who were previously asymptomatic. We recommend routine screening, although this may be challenging, both because of its cost-effectiveness and technical difficulties. G6PD screens can be falsely negative during acute haemolytic episodes and when the testing is done after blood transfusion, as donor red blood cells can mask G6PD deficiency. White cells contain a significant amount of G6PD, and ideally, they should be removed prior to assay, especially if the count is above the lab's reference interval upper limit which is often the case in leukaemia. Furthermore, it can be logistically difficult to delay rasburicase therapy because of the high morbidity and mortality associated with delayed treatment of TLS and variability in turnaround time of G6PD screening results.

Differentiation syndrome (DS)

Differentiation syndrome (DS), also known as retinoic acid syndrome, is the main life-threatening complication of therapy with differentiating agents, all-*trans* retinoic acid (ATRA, tretinoin) or arsenic trioxide, in patients with acute promyelocytic leukaemia. It typically occurs during induction therapy with differentiating agents, especially in patients presenting with high white blood cell count at diagnosis or relapsed disease. The median day of onset of clinical manifestations of the DS ranges between 7 and 12 days after starting therapy with differentiating agents.

This syndrome is characterised by unexplained fever, weight gain, hypotension, acute respiratory distress with interstitial pulmonary infiltrates and/or a vascular capillary leak syndrome leading to acute renal failure. The diagnosis of DS is mainly based on clinical and radiological features; however, it usually requires the exclusion of alternative causes that could explain the signs and symptoms of the syndrome. The early addition of chemotherapy to ATRA and the administration of high doses of steroids in the form of dexamethasone at the onset of the first sign or symptom has reduced the mortality associated with this syndrome from 30% down to 5% or less. It is recommended to start treatment with intravenous dexamethasone at a dose of 10 mg twice daily promptly at the very earliest suspicion of DS. Most patients will show rapid improvement and complete resolution of signs and symptoms after starting intravenous dexamethasone. Corticosteroid therapy should be continued until the complete disappearance of symptoms and then tapered. Other supportive measures are crucial in the correct management of DS. Furosemide is usually administered to treat signs or symptoms of fluid overload. Some cases with refractory acute renal failure and/or fluid overload may need renal replacement therapy. Invasive or non-invasive mechanical ventilation is indicated in some patients with severe acute respiratory failure who do not respond to high-flow oxygen therapy. Patients with prerenal failure and hypotension require careful fluid monitoring in conjunction with inotropic support.

Temporary discontinuation of ATRA or ATO is recommended in the cases of DS with very poor clinical conditions or severe organ dysfunction, such as patients developing renal failure or requiring mechanical ventilation because of respiratory distress. Otherwise, these differentiating agents could be maintained unless progression to overt syndrome or lack of response to dexamethasone is observed. If a favourable response is obtained, dexamethasone should be maintained until complete disappearance of signs and symptoms, and then ATRA or ATO should be resumed.

Nutrition and physiotherapy

High dose therapies, particularly haematopoietic cell transplantation have marked effects on metabolic demands and significantly impair nutritional intake. Treatment associated side-effects such as severe mucositis, nausea and vomiting can lead to rapid deterioration of nutritional status. Furthermore, reduced physical activity and loss of mobility due to hospitalization and prolonged isolation result in reduced muscle mass, particularly in a previously active patient. Nutrition support alongside of physiotherapy are therefore recognised as an important adjunct to high-dose therapy.

Optimal nutrition may improve tolerance to treatment and outcome in malnourished patients and well-nourished individuals may experience less treatment delays, myelosuppression and infections. Studies in transplantation showed association between poor nutritional status and low body mass index (BMI) with longer time to engraftment and lower survival. Conversely, high BMI prior to HCT is linked with acute GvHD, infections and poorer 5-year survival rates. Although association is clear, causality is difficult to establish in clinical studies.

Damage to the gut mucosal barrier by high-dose therapy, bacterial overgrowth and opioid-related impairment of peristalsis has raised concerns regarding the risk of bacterial translocation, leading to recommendations to follow a 'neutropenic diet'. Small studies have not confirmed the benefit of excluding uncooked fruit and vegetables or following a low-bacterial diet. More recently, a large retrospective study suggested less infections when neutropenic dietary restrictions were removed (Trifilio *et al*. 2012). Overall, there is lack of evidence for a relationship between dietary intake and infections during high-dose therapy. Any advice given with the intention of minimising foodborne infection should focus on hygienic practices and prioritise nutritional status over theoretical risks.

Artificial nutrition is indicated where a patient undergoing therapy cannot meet their needs with oral intake. The metabolic, physiologic and economic advantages of enteral over parenteral nutrition are well established. In the allogeneic transplantation setting, enteral nutrition has demonstrated an association with lower incidence of overall and gut acute GvHD than parenteral nutrition (Beckerson *et al*. 2019), perhaps because of its ability to maintain mucosal integrity, modulate the immune response to intensive chemo/radiotherapy and support the gastrointestinal tract environment, including intestinal microbiota.

Mineral and electrolyte losses are common during chemotherapy and are often exacerbated by antimicrobial and immuno-suppressive drugs (amphotericin, ciclosporin). Losses of fluid, electrolytes and other nutrients frequently occur. Intravenous input from medications, blood products and electrolyte replacement can predispose to fluid overload. Micronutrient requirements may be raised during high-dose therapy due to increased oxidation and tissue damage however there is no evidence to support routine antioxidant or trace element supplementation. Multiple blood transfusions may elevate ferritin levels indicating iron overload. Vitamin D has a role in normal haematopoiesis and modulation of the immune system. Some evidence points to potential benefits in the treatment of

myeloid disorders and suggests the vitamin D receptor has an impact on immune reconstitution after haematopoietic cell transplantation and hence subsequent risks of infection, graft versus host disease and graft versus disease (Hall and Juckett 2013).

Repeated hospital stays for treatment can result in suboptimal vitamin D status and low calcium levels which are of particular relevance for those receiving corticosteroid therapy. In the longer term, continued hospital appointments, fatigue and susceptibility to infection significantly impact nutrition and rehabilitation after high-dose therapy. Full return to normal functioning and nutritional status can take more than 1 year after treatment is completed.

Chemotherapy-induced nausea and vomiting (CINV)

Nausea and vomiting are common in patients undergoing treatment for leukaemia. Both symptoms are distressing, cause significant morbidity and reduce quality of life. Nausea and vomiting contribute significantly to nutritional deterioration and increased pain intensity. The emetogenicity of the chemotherapeutic agents, repeated chemotherapy cycles and patient risk factors significantly influence CINV. The use of a combination of a serotonin 5-HT3 receptor antagonist, dexamethasone and a neurokinin 1 (NK1) receptor antagonist has significantly improved the control of acute and delayed emesis in single-day chemotherapy. Palonosetron, a second-generation 5-HT3 receptor antagonist with a different half-life, a different binding capacity and a different mechanism of action from the first-generation 5-HT3 receptor antagonists appears to be the most effective agent in its class. Aprepitant, the first and only agent clinically available in the NK1 receptor antagonist drug class has been used effectively as an additive agent to the 5-HT3 receptor antagonists and dexamethasone to control CINV. Rolapitant and netupitant are other NK1 receptor antagonists that are currently in Phase III clinical trials. Olanzapine, a US-FDA-approved antipsychotic, has emerged in recent trials as an effective preventative agent for CINV, as well as a very effective agent for the treatment of breakthrough emesis and nausea.

Clinical trials using gabapentin, cannabinoids and ginger have not been definitive regarding their efficacy in the prevention of CINV. Additional studies are necessary for the control of nausea and for the control of CINV in the clinical settings of multiple-day chemotherapy and bone marrow transplantation.

Non-pharmacological strategies are very important in the overall management of the patient. Examples include avoiding food with strong tastes and smells; small but frequent meals, behavioural approaches (e.g. relaxation) and acupuncture/reflexology.

Pain control and palliative care

Many leukaemia patients experience bone or joint pain that results from the bone marrow being heavily infiltrated by leukaemia cells. Oral and gut mucositis are probably the most common site of pain, especially in recipients of high-dose chemotherapy or total body irradiation. Infections can cause pain, especially in areas such as the skin, mouth, eyes and perianally. Avascular necrosis of bone is another cause of pain in patients with acute lymphoblastic leukaemia and is often severe enough to need relief with opiates. Peripheral neuropathy and associated neuropathic pain are a widespread side-effect of treatment with certain chemotherapeutic agents. The pain associated with these neurotoxic effects can be prolonged, severe and relatively resistant to intervention. The overall incidence is not clearly delineated, although it is documented frequently with vincristine and platinum-based agents.

A variety of modalities to treat and control pain include:

- Pain control drugs with non-opioid and opioid analgesics
- Neuropathic pain control drugs such as carbamazepine, gabapentin, and antidepressants (amitriptyline, nortriptyline and imipramine)
- Continuous pump infusions
- Nerve block therapies
- Psychosocial interventions – stress management, counselling and coping mechanisms
- Acupuncture
- Massage therapy
- Relaxation techniques.

Palliative care

Relapse post-chemotherapy or following a bone marrow transplant is a common theme in acute leukaemia. Intensive salvage chemotherapy or other modalities can often achieve prolonged remission; however, refractory unresponsive-to-chemotherapy disease is not uncommon in this setting. Several experimental treatments can be offered; however, it is important to weigh the possible limited benefits of a new treatment against the possible downsides as quite often further treatment might not improve the health or change the outcome or survival. Under these circumstances, palliation can be offered to the patient. Palliative care is a proactive approach involving a multi-professional team. As well as controlling pain and other distressing symptoms, it applies a holistic approach to meeting the physical, practical, functional, social, emotional and spiritual needs of patients and carers facing progressive illness and bereavement. It is not uncommon for patients to ask to remain under the care of the same haematology team; however, the palliative care team should be involved as the choice of dying at home versus dying in a hospice requires expertise and external support is better managed by palliative care services.

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CHAPTER 56

General consultative haematology

56

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Cancer

Anaemia

Anaemia is the most common haematological disturbance in patients with cancer. This may arise due to a number of mechanisms and, in common with other anaemias, it can be broadly categorised as being due to either reduced production of red cells (Table 56.1), increased destruction (Table 56.2), or increased blood loss. In many patients with malignancy, the anaemia arises due to a combination of several factors, and identifying and addressing each of these may lead to a rise in the haemoglobin level potentially ameliorating the patient's symptoms of fatigue.

Anaemia due to reduced red cell production

Haematinic deficiencies can arise in patients with malignancy, especially those undergoing chemotherapy or radiotherapy, due to reduced oral intake or reduced absorption of nutrients. Systemic anti-neoplastic treatments themselves can be directly myelosuppressive, inhibiting erythropoiesis and contributing to anaemia.

Tumours, or treatments that lead to a deterioration in renal function, can directly lead to reduced levels of erythropoietin, though more often in patients with cancer this hormone is supressed in association with an anaemia of inflammation/chronic disease (see Chapter 11), with raised inflammatory markers. Exogenous erythropoietin supplementation may be beneficial in this circumstance to help correct the anaemia, and reduce fatigue.

Infiltration of the bone marrow by malignancy may directly cause anaemia and is most common with haematological malignancies, such as myeloid disorders, and myeloma. It can also occur with metastatic spread of other primary malignancies, especially from the prostate, breast or the gastrointestinal tract where it is more likely to lead to a leucoerythroblastic peripheral blood smear, characterised by the presence of myeloid precursors, nucleated red blood cells, and teardrop cells. More rarely the bone marrow can be directly infiltrated by amyloidomas which can displace normal haematopoietic activity.

Pure red cell aplasia refers to the failure of erythropoiesis only and is characterised by a normocytic anaemia with reticulocytopenia in the absence of other causes. This can occur in association with certain malignancies, most commonly haematological, such as chronic lymphocytic leukaemia (CLL), large granular lymphocytic (LGL) leukaemia, and non-Hodgkin lymphomas, but it can also occur in association with thymomas. In immunosuppressed individuals, chronic infection with parvovirus (erythrovirus) B19 will lead to pure red cell aplasia, and serology and PCR testing can be performed to investigate this possibility.

Anaemia due to increased red cell destruction

Premature red cell destruction (defined as a red cell life span of less than 100 days) can occur through either immune, or non-immune mediated haemolysis (see also Chapter 17) in patients with malignancy (Table 56.2).

Immune haemolysis

The production of antibodies leading to auto-immune haemolytic anaemia (AIHA) may occur in association with malignancy,

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Companion website: www.wiley.com/go/mead/HPH8e

Table 56.1 Causes of reduced red cell production in patients with cancer.

Cause of reduced red cell production	Notes
Anaemia of chronic disease	Low transferrin and transferrin saturations, with raised inflammatory markers
Haematinic deficiency – iron	Microcytosis. Ferritin may be normal or high in patients with cancer. Characterised by low transferrin saturations.
Haematinic deficiency – B12	Typically takes years to develop, but may occur in patient with previous surgical resection of the stomach (the source of intrinsic factor production), or terminal ileum (site of absorption)
Haematinic deficiency – folate	Folate is the haematinic store first to be depleted due to poor oral intake
Cytotoxic chemotherapy agents	Can cause direct marrow suppression
Renal anaemia	Renal impairment with reduced erythropoietin levels
Direct marrow infiltration	Most commonly by haematological malignancy or amyloid deposition
Pure red cell aplasia – direct cancer effect	Especially in conjunction with chronic lymphocytic leukaemia, T-cell large granular lymphocytic leukaemia, non-Hodgkin lymphoma. Also seen in association with thymomas
Pure red cell aplasia – viral induced	Parvovirus (erythrovirus) B19

Table 56.2 Causes of increased red cell destruction in patients with cancer.

Cause of increased red cell destruction	Notes
Autoimmune haemolytic anaemia	Most often associated with chronic lymphocytic leukaemia and other haematological malignancies
Microangiopathic Haemolytic Anaemia – cancer-associated	Typically associated with mucinous adenocarcinomas
Microangiopathic Haemolytic Anaemia – therapy-associated	Ciclosporin, tacrolimus, mitomycin and platinum-based chemotherapy
Haemophagocytic Lymphohistiocytosis	Most often associated with haematological malignancies (lymphomas and leukaemias), but occasionally with solid organ cancers.

especially haematological. This is characterised by a positive direct Coombs' test, raised bilirubin and LDH, and reduced haptoglobin. The haematological cancer most frequently associated with AIHA is chronic lymphocytic leukaemia, but it can complicate other B-cell non-Hodgkin lymphomas, T-cell lymphomas, myeloid malignancies, and less commonly can occur in association with non-haematological malignancies. Most often AIHA arising in association with malignancy is warm-type, however cold agglutination haemolytic anaemia may arise, most often in association with lymphoplasmacytic lymphoma, CLL or other lymphomas.

Non-immune haemolysis

Premature red cell destruction can occur through a number of non-immune mediated mechanisms in patients with malignancy. A microangiopathic haemolytic anaemia (MAHA) can arise in association with certain cancers, most often mucinous adenocarcinomas of the stomach, breast and lung. This will be characterised by a negative direct Coombs' test, a fall in platelets, the presence of red cell fragments on a peripheral blood smear, and an elevation in LDH. Certain medications may precipitate MAHA, including ciclosporin, tacrolimus, mitomycin and platinum-based chemotherapy.

Haemophagocytic Lymphohistiocytosis (HLH) (see also Chapter 36) is a condition in which there is phagocytosis of red cells, either by macrophages, or sometimes by tumour cells themselves. It is typified by profound elevations in the ferritin level, cytopenias, splenomegaly and persistent fevers which are not responsive to anti-microbial agents. This condition can occur as a secondary complication of certain malignancies, most often lymphoma or leukaemia, and there is often an infectious trigger, most typically EBV infection.

Increased blood loss

The anatomical abnormalities that arise within a tumour can predispose to bleeding, most commonly intra-luminal in patients with gastrointestinal, or genitourinary tumours. Less commonly bleeding can occur within a tumour, for example hepatocellular carcinoma, or into body cavities such as peritoneal or retroperitoneal bleeding.

Malignancies and their treatment can cause the development of thrombocytopenia, or abnormalities of haemostasis such as disseminated intravascular coagulopathy which in turn will increase the tendency to more generalised bleeding problems.

Polycythaemia

Polycythaemia occurs in certain myeloproliferative neoplasms as a direct consequence of genetic dysregulation of the bone marrow, and in most cases, an underlying genetic mutation can be identified from analysis of the peripheral blood (see Chapter 35).

Non-haematological malignancy associated polycythaemia is rare and is generally caused by secretion from the tumour of erythropoietin, or erythropoietin-like peptides, causing an increased red cell production. This is most often associated with renal cell carcinoma or hepatoma, but can also occur in patients with uterine myoma, androgen-secreting ovarian tumours, pheochromocytoma and cerebellar haemangioblastoma.

Leucocytes

The white cell count is frequently perturbed in patients with haematological and non-haematological cancers. Malignancy will often cause a neutrophilia to develop, driven by the inflammatory state and by increased production of cytokines. Cytotoxic chemotherapy or widespread radiotherapy will typically suppress neutrophil and other white cell levels. Lymphopenia is a common finding after chemotherapy and may be chronic.

Whilst most changes in white cell levels in association with malignancy are non-specific, there are a few common associations that should be noted (Table 56.3).

Platelets

Thrombocytosis

It is common for platelet levels to be elevated in association with malignancy. In myeloproliferative neoplasms, this is due to a primary bone marrow pathology (Chapter 35). In other malignancies, it is a reactive phenomenon, driven by chronic inflammatory changes, or in association with iron deficiency, or both.

Thrombocytopenia

There are many causes of thrombocytopenia in association with malignancy (Table 56.4). Direct infiltration of the bone marrow, typically by haematological malignancies will often be associated

Table 56.3 Leucocyte changes associated with malignancy.

White cell change	Association
Neutrophilia	Myeloproliferative conditions Most malignancies Infections that may occur in the context of malignancy and its treatment
Neutropenia	Myeloid malignancies, T-cell large granular lymphocytic leukaemia, bone marrow infiltration by malignancy or as a consequence of chemo/radiotherapy
Monocytosis	Chronic myelomonocytic leukaemia, Hodgkin lymphoma
Monocytopenia	Hairy cell leukaemia, or as a consequence of chemo/radiotherapy
Lymphocytosis	Chronic Lymphocytic leukaemia, non-Hodgkin lymphoma or other lymphoproliferative disorders. Also seen post-splenectomy
Lymphopenia	Most common following systemic chemotherapy, with steroids, or in association with opportunistic infection
Basophilia	Myeloproliferative disorders
Eosinophilia	Hodgkin lymphoma, T-cell lymphomas, myeloproliferative disorders. Also as part of an allergic reaction to medication, or in association with opportunistic infection

Table 56.4 Causes of thrombocytopenia in patients with malignancy.

Cause of low platelets in association with malignancy	Association
Primary bone marrow pathology	Myeloid disorder, such as myelodysplasia or acute myeloid leukaemia
Bone marrow infiltration	Myeloma, infiltration of marrow by non-haematological malignancy
Suppression of bone marrow function	By chemo/radiotherapy
Increased platelet destruction	Microangiopathic haemolytic anaemia, Disseminated intravascular coagulation, Thrombotic thrombocytopenic purpura
Increased platelet destruction – immune	May occur in association with CLL, other lymphomas or myelodysplasia, and rarely with non-haematological malignancies
Splenic pooling	Occurs in any cause of splenic enlargement, either directly due to malignancies such as myelofibrosis, or due to liver pathologies that cause raised portal vein pressures

with a reduced peripheral blood platelet count. Progression of myeloma may also correlate with a falling platelet count.

The cytotoxic effects of many chemotherapy agents will cause a drop in platelets over several days, due to the combination of reduced marrow production with the short half-life of platelets. Widespread radiotherapy will have a similar effect.

Increased platelet destruction occurs in a number of systemic conditions associated with malignancy, including MAHA, disseminated intravascular coagulation and thrombotic thrombocytopenic purpura. Platelet destruction may also occur through immune mechanisms, most commonly in association with chronic lymphocytic leukaemia, though less frequently this can be seen in patients with lymphoma. Immune platelet destruction is occasionally associated with non-haematological malignancies.

Certain cancers will cause an increase in the size of the spleen, and this will result in increased sequestration of platelets in the spleen, and lower numbers circulating in the peripheral blood. Primary haematological malignancies causing splenomegaly include the myeloproliferative neoplasms (especially myelofibrosis or chronic myeloid leukaemia), and splenic marginal zone lymphomas. Non-haematological cancers that increase the pressures in the portal vein resulting in splenomegaly will also cause a reduction in the circulating platelet count.

Other systemic diseases

Renal disease

Anaemia

Anaemia is the most common haematological manifestation of renal disease (Table 56.5), with different mechanisms underlying its development in either acute or chronic renal impairment.

When anaemia occurs in conjunction with acute renal impairment, it is typically caused directly by the same pathology that is affecting the kidneys, and management of the underlying cause is required to ameliorate the anaemia.

In chronic renal impairment, regardless of aetiology, anaemia most often develops secondary to inappropriately low levels of erythropoietin secretion by the damaged kidneys, but other causes can also contribute (Table 56.6). Typically red cells are normocytic and normochromic, with an inappropriately low reticulocyte count. Bone marrow investigation would show normoblastic erythropoiesis, without adaptive hyperplasia of the erythroid lineage. In a population-based sample of over 15,000 patients, anaemia was seen in 1% of individuals with an estimated glomerular filtration rate (eGFR) of 60 mL/min, 9% of individuals with an eGFR of 30 mL/min, and 33% (men) to 67% (women) of those with an eGFR of 15 mL/min. Replacement of erythropoietin, typically sub-cutaneously, is usually effective in improving red cell levels, and quality of life. The dose should be titrated, aiming for a haemoglobin level of 100–120 g/L, as higher levels are associated with an increased risk of thrombosis. Blood pressure should be monitored as hypertension is common. Long-acting erythropoietin replacement allows administration weekly, or bi-weekly, rather than 3 times a week with the standard formulation. Patients under consideration for exogenous erythropoietin will often benefit from intravenous iron replacement, as deficiency of iron, often functional and due to disturbances in the mobilisation of iron, is common, and will impair the response to erythropoietin. Low transferrin saturations may be a better guide to the need for iron replacement than ferritin, which is often elevated by the inflammation associated with chronic disease.

Polycythaemia

Renal disease can be associated with polycythaemia, usually through inappropriately high levels of erythropoietin production. This phenomenon is found in up to 5% of renal cell carcinomas and up to 15% of patients following renal transplantation, where haematopoietic factors such as insulin-like growth factor 1 may also increase sensitivity to erythropoietin. Localised renal hypoxia, for example in renal artery stenosis, may also drive inappropriate erythropoietin release.

Table 56.5 Causes of anaemia in acute renal impairment.

Causes of anaemia in acute renal impairment	Notes
Microangiopathic haemolytic anaemia, Haemolytic-Uraemic syndrome Thrombotic thrombocytopenic purpura	Red cell fragments will be seen on the blood film, and there will be a reduction in platelet number. LDH is elevated. Typically in association with bacterial gastroenteritis, most often E-Coli O157 May be associated with fever, neurological and cardiac abnormalities. ADAMTS13 levels will be low
Disseminated intravascular coagulation	Most often caused by infection, but can occur in association with malignancy
Systemic infection	Renal impairment may be driven by dehydration, drugs and radiological investigation. Anaemia develops via increased red cell destruction and raised hepcidin levels

Table 56.6 Causes of anaemia in chronic renal impairment.

Causes of anaemia in chronic renal impairment	Notes
Inappropriately low erythropoietin	Normocytic, normochromic anaemia with inappropriately low erythropoietin and reticulocyte count.
Haematinic deficiency – Iron	Ferritin may be normal or high in the presence of underlying inflammation even with iron deficiency. Ferritin <200 ng/mL in a dialysis patient would suggest iron deficiency. Transferrin saturations will be low. Intravenous replacement of iron may be more efficacious than oral.
Haematinic deficiency – Folate	Folate can become depleted by dialysis and should be replaced pre-emptively
Medication-induced	Medications used in the treatment of renal disease, or in the prevention of organ rejection post-transplant may cause anaemia, for example immunosuppressants such as mycophenolate or tacrolimus
Myeloma	Myeloma can cause anaemia directly through marrow involvement. Renal impairment can occur through multiple modalities, including direct light chain toxicity, hypercalcaemia, and through medications.
Anaemia of chronic disease	Hepcidin levels are usually elevated in patients with chronic kidney disease, due to chronic inflammation and reduced renal excretion
Increased blood loss	As uraemia increases, so too does the tendency to bleeding, further exacerbating anaemia.
Aluminium toxicity	Aluminium is not efficiently removed by dialysis and may suppress erythropoiesis if levels rise, but aluminium-free dialysis fluids and phosphate binders in common use today make this a rare cause of anaemia.

Liver disease

Liver disease causes a greater range of haematological change than does disease in any organ, other than the bone marrow. The liver is an important source of erythropoietin in the fetus, and serves as a haemopoietic organ *in utero*; extramedullary haemopoiesis occurs within the adult liver only in pathological states (e.g. primary myelofibrosis, severe haemolysis or megaloblastic anaemia). The liver is the major source of thrombopoietin in adults.

Platelets

Patients with liver disease may have abnormalities of their platelet count (Table 56.7). Thrombocytopenia is the most common haematological abnormality in chronic liver disease, and a slowly progressive fall in platelets is often the first sign of such a disease emerging. Patients, even with severe cirrhosis rarely have a platelet count less than $30–50 \times 10^9$/L.

Historically, the low platelet count was attributed to congestive splenomegaly, leading to sequestration in the spleen, and whilst this is undoubtably an important contributor to thrombocytopenia, there are multiple additional mechanisms involved. Lower levels of thrombopoietin are seen in patients with chronic liver disease, and bone marrow function may be supressed, for example by alcohol or viruses, leading to reduced platelet production. There may also be reduced lifespan of these cells, due to increased activation, or by auto-immune destruction.

Table 56.7 Platelets and liver disease.

Changes in platelets in liver disease
Thrombocytopenia
Splenic pooling
Reduced levels of thrombopoietin
Bone marrow suppression, for example by viruses or alcohol
Increased platelet destruction, for example by increased shear forces, or auto-immune processes
Thrombocytosis
Rebound thrombocytosis following alcohol cessation, in combination with improved nutrition
Hepatocellular carcinoma – can secrete thrombopoietin as paraneoplastic phenomenon

Thrombocytosis is less common in liver disease, but can occur following cessation of chronic alcohol, especially when combined with improved nutritional intake. This phenomenon usually lasts up to 2 weeks before self-correcting, and is a normal physiological response that does not require treatment.

Anaemia

Anaemia in liver disease is common (Table 56.8), and generally multifactorial, with reduced production, increased destruction, blood loss and sequestration all contributing.

Table 56.8 Causes of anaemia in liver disease.

Bleeding
Haematinic deficiency – iron
Haematinic deficiency – folic acid
Haemolysis
Zieve syndrome
Anaemia of chronic disease
Alcohol-induced sideroblastic anaemia
Splenic sequestration
Alcohol-induced bone marrow toxicity
Wilson disease – may cause a Coombs' test negative haemolytic anaemia due to direct toxicity of copper ions on red cells
Viral liver disease – see section on infective diseases

Reduced production of red cells may occur due to the anaemia of chronic disease, haematinic deficiency, or via the toxic effects of medications used to treat the underlying liver disease. Acute or chronic alcohol toxicity, which may accompany liver disease, can directly cause impairment of erythropoiesis within the bone marrow.

Liver disease of any aetiology, especially alcohol-related, may lead to the development of a macrocytosis. Increased cholesterol and phospholipid content in the membrane bilayer leads to overproduction of red cell membrane and the subsequent appearance of target cells on the peripheral blood film. In more advanced liver disease, the cholesterol content of the membrane increases further, and acanthocytes (cells with 2–10 thorn-like asymmetric protrusions) may be seen, which can be associated with non-immune haemolysis ('Spur cell anaemia') especially in alcoholic cirrhosis. Stomatocytes (red cells with a slit of central clearing) may be seen in severe liver disease, especially alcohol-related. Acanthocytes should not be confused with the more commonly seen echinocytes (red cells with multiple, smaller, and more regularly distributed membrane projections). These cells are less specific for liver disease, also being seen in severe renal impairment, and are not thought to have a shortened survival. Echinocytes are thought to occur due to the deposition of abnormal high-density lipoproteins from the plasma onto the red cell membrane.

Mild haemolysis is common due to the acquired membrane defects of chronic liver disease. Zieve syndrome is a rare disorder typically occurring after an acute episode of increased alcohol intake in patients with alcoholic liver disease. It is characterised by a triad of non-immune haemolytic anaemia, cholestatic jaundice and hypertriglyceridaemia.

Increased blood loss is a common cause of anaemia in liver disease, due to a number of mechanisms. Portal hypertension may lead to the development of varices, promoting both episodic, as well as chronic blood loss. Splenomegaly leads to thrombocytopenia by sequestration of platelets and, combined with defective haemostasis due to reduced liver synthetic function, potentially accentuates bleeding. Cirrhosis of the liver may cause hypersplenism, leading to the sequestration of red cells, as well as increased clearance and haemolysis.

Polycythaemia

Hepatocellular carcinoma is associated with raised erythropoietin levels in up to 25% of cases, however, only rarely does this result in polycythaemia as its effect is usually modulated by anaemia of chronic disease or abnormal bleeding.

White cells

Neutrophilia may be seen in association with liver disease complicated by malignancy, haemorrhage, haemolysis, infection or when corticosteroids are being administered. Neutropenia may be seen in patients with hypersplenism.

Orthotopic liver transplantation

Thrombocytopenia is frequently present prior to transplantation. The count tends to fall postoperatively, despite platelet transfusions and this may be due to platelet sequestration in the transplanted liver. Immune thrombocytopenia has also been reported after orthotopic liver transplantation (OLT). Antibody-mediated haemolysis occurs in recipients of ABO-incompatible grafts, but the engrafted liver can also produce mild haemolysis due to anti-recipient ABO antibody. This is a form of humoral graft-versus-host disease (GVHD), but T cell-mediated GVHD has also been reported after OLT. Although aplastic anaemia is a rare complication of viral hepatitis, it may occur in as many as 30% of patients transplanted for fulminant non-A, non-B viral hepatitis.

Connective tissue disorders

Haematological manifestations of connective tissue disorders are very common, typically arising as a result of inflammation, auto-immune processes or drug toxicities. In most cases, therapy should be directed at better control of the underlying rheumatological disorder.

Anaemia

Anaemia frequently complicates connective tissue disorders (Table 56.9). In rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) this is usually due to the anaemia of chronic disease but can be compounded by additional mechanisms.

Long-term use of non-steroidal anti-inflammatory medications, for example by a patient with RA to control joint pain, often causes intestinal inflammation, and frequently iron deficiency due to blood loss which may not be noted by the patient.

Auto-immune haematological complications are common. Warm or cold AIHA, with a direct Coombs' test positive for IgG

Table 56.9 Anaemia in connective tissue disorders.

Anaemia of chronic disease
• With raised hepcidin levels
Blood loss +/− iron deficiency, for example through NSAID-induced intestinal inflammation
Haematinic deficiency – folic acid
Auto-immune haemolysis
• Direct antigen test positive for IgG (Warm), complement (Cold) or both (mixed)
Pure red cell aplasia
• Which may be accompanied by reticulocytopenia
Direct marrow toxicity of medications
Micro-angiopathic haemolytic anaemia – increased risk of thrombotic thrombocytopenic purpura, catastrophic anti-phospholipid syndrome or disseminated intravascular coagulopathy
• Red cell fragmentation, reduced platelet count and elevated LDH are seen.
Haemophagocytic lymphohistiocytosis/macrophage activation syndrome (see Chapter 36)
• Marked elevation of ferritin, often to greater than 10,000 µg/L is seen.

and/or complement, is a frequent occurrence in the connective tissue disorders, especially SLE where it occurs in 10% of patients, and RA. Evans syndrome, the combination of AIHA with ITP, occasionally also with auto-immune neutropenia, can be seen in patients with SLE. Pure red cell aplasia and reticulocytopenia may occur if antibodies are directed against early red cell precursors within the marrow.

Medications used in the treatment of these diseases, such as hydroxychloroquine, mycophenolate and cyclophosphamide may all suppress bone marrow function, whereas methotrexate and azathioprine can cause a megaloblastic anaemia.

White cells

Change in white cells, either reduced, or increased are common in patients with connective tissue disorders.

Neutropenia

Neutropenia and lymphopenia are both common and are usually mild in patients with connective tissue disorders.

Felty syndrome is the triad of neutropenia and splenomegaly in a patient with rheumatoid arthritis, and occurs in approximately 1% of patients with RA, typically those with severe chronic disease. Splenomegaly is not always seen, and treatment is typically with methotrexate, rituximab or steroids.

T-cell large granular lymphocytic leukaemia (T-LGL) is associated with RA in a third of cases, but can also occur in patients with Sjögren syndrome, or SLE. It presents with neutropenia in the majority of patients, as well as with an increased proportion of circulating large granular lymphocytes on the blood film. Since splenomegaly may also be seen, it may mimic Felty syndrome, and is distinguished by the identification of clonal T-cells, and on bone marrow examination, where heavy lymphocytic infiltration is seen. Unlike Felty syndrome, it may present in patients with any degree of connective tissue disorder, whereas Felty syndrome more typically occurs in those with severe disease. Treatment is with low-dose immunosuppression, typically ciclosporin or methotrexate.

Drug-induced neutropenia may be seen, and occasionally agranulocytosis, which is rarely associated with non-steroidal anti-inflammatory medications and gold salts.

Macrophage activation syndrome may occur, especially in patients with SLE, and should be considered in patients with cytopenia, unexplained fever, and very high ferritin levels (see Chapter 36).

Neutrophilia

A raised neutrophil count is common when connective tissue disorders are active, or if corticosteroids are being used in their treatment. Evidence of bacterial infection should be sought, especially in the unwell patient, or those on immunosuppressive medications.

Eosinophilia

This may complicate RA, SLE as well as the vasculitides, most commonly eosinophilic granulomatosis with polyangiitis (formally known as Churg-Strauss syndrome). This latter condition, which may affect any organ, is most often characterised by asthma which is difficult to control, chronic rhinitis, recurrent sinusitis, and eosinophilia of greater than 1.5×10^9/L. An eosinophilic infiltrate is generally seen on biopsies, along with vasculitis and necrosis.

Platelets

Immune-mediated thrombocytopenia (ITP) is the most common cause of a severe reduction in platelets in patients with connective tissue disorders and is seen in up to half of patients with SLE, where it is usually mild, and its onset may precede the diagnosis of SLE. ITP is also associated with RA and other connective tissue disorders, although less commonly.

Thrombocytosis, as a reactive phenomenon, is also seen frequently in the connective tissue disorders, and the extent of the platelet elevation often correlates with disease activity.

Congestive cardiac failure (CCF)

Anaemia is very commonly seen in patients with congestive cardiac failure (CCF), where its cause is usually multifactorial. Anaemia of chronic disease, haemodilution, medications and co-existent renal impairment may all contribute, and haematinic

deficiency should be excluded. Venous congestion may lead to the development of hypersplenism, with associated sequestration and destruction of platelets, although this is usually mild.

Endocrine disorders

A variety of changes to red cells and white cells can be seen in patients with endocrine disorders (Table 56.10). Anaemia may be seen in either hypothyroidism (typically normocytic or macrocytic), or hyperthyroidism (usually normocytic, and may be associated with a mild neutrophilia). In both disorders, there tends to be an increase in fluid retention, such that the anaemia is partially dilutional. Thyroid hormones stimulate erythropoiesis, so patients with hypothyroidism typically develop an anaemia which may be worsened by associated increases in menorrhagia and reduced iron absorption and utilisation (in part due to increased achlorhydria). Other causes of anaemia in patients with hypothyroidism include pernicious anaemia, which is also more common in patients with hypoadrenalism or hypoparathyroidism. Medications used in the treatment of hyperthyroidism (for example carbimazole and propylthiouracil) can cause aplastic anaemia or agranulocytosis.

Anaemia may be seen in patients with diabetes mellitus, often due to co-existent renal disease, anaemia of chronic disease, or as a side-effect of medications.

Table 56.10 Haematological consequences of endocrine disease.

Red cells
Thyrotoxicosis → anaemia (normochromic and usually normocytic)
Hypothyroidism → anaemia (normochromic, normocytic, occasionally macrocytic)
Diabetes mellitus → anaemia (usually when complicated by renal failure, infection, cardiac disease or enteropathy)
Hyperparathyroidism → anaemia (normochromic, normocytic)
Hypoadrenalism → anaemia (normochromic, normocytic)
Hypogonadism → anaemia (normochromic, normocytic)
Hypopituitarism → anaemia (normochromic, normocytic)
Phaeochromocytoma → Anaemia (normochromic, normocytic) or more rarely polycythaemia
Cushing syndrome → Polycythaemia by the stimulation of erythropoiesis by corticosteroids

White cells
Cushing syndrome → Neutrophilia
Phaeochromocytoma → Neutrophilia
Antithyroid drugs → Neutropenia
Diabetes mellitus → Impaired neutrophil function
Hyperthyroidism → lymphocytosis

Effect of body mass index on full blood count

A chronic, mild, leucocytosis and thrombocytosis may be seen in patients with an elevated body mass index (typically greater than 30 kg/m^2), although investigation and exclusion of other causes may be necessary depending on findings from the patient's history, examination and other blood results.

A hypoxia-driven secondary polycythaemia may develop in patients with raised body mass index, due to obstructive sleep apnoea. Patients should be screened for this condition with an Epworth Sleepiness Scale questionnaire and may require sleep studies to confirm the diagnosis.

Patients with low body weight due to anorexia, or starvation, may develop cytopenias, which generally worsen with the severity of their disease. Anaemia (normochromic, normocytic) and leukopenia, typically mixed lymphopenia and neutropenia both occur commonly (33% of outpatients, and up to 83% of in-patients). Thrombocytopenia is less common (10% of outpatients, and up to 25% of in-patients) and severe thrombocytopenia is rare. Bone marrow biopsy is hypocellular, with gelatinous deposits (serous atrophy) commonly observed. Evidence of haematinic deficiency should be sought, but recovery of bone marrow function is generally achieved only with restoration of adequate nutrition and weight.

Environmental, nutritional and age-related changes to full blood count

Other systemic causes of leukocytosis

Smoking may cause mild neutrophilia, with the degree of elevation linked to the number of cigarettes smoked per day. Polycythaemia may also be seen, driven either by hypoxia related to smoking-related pulmonary disorders or due to a reduced plasma volume caused by increased capillary permeability.

Asplenism (either surgical or via auto-infarction for example in sickling disorders) typically causes a moderate neutrophilia, often accompanied by a mild thrombocytosis. Howell-Jolly bodies and occasional nucleated red blood cells can be seen on the blood smear. Such patients often demonstrate an exaggerated white cell response (a leukaemoid reaction) to infection or inflammation.

Copper deficiency

Copper deficiency may occur due to malabsorption in patients who have had gastric bypass surgery or a previous gastrectomy, or those with coeliac disease, or due to excess zinc ingestion, for instance in denture creams or supplements, which out-competes copper for absorption in the gut. It causes

anaemia which is usually normocytic and may be severe and neutropenia which is often less than 1×10^9/L. Thrombocytopenia is rare. Diagnosis is by detection of low serum copper and caeruloplasmin levels. Bone marrow examination may mimic sideroblastic anaemia as ring sideroblasts may be seen, with increased haemosiderin.

Aging and anaemia

Anaemia is more commonly observed with increasing age. This may be due to the increased prevalence of renal dysfunction, increasing co-morbidity and tendency to development of anaemia of chronic disease. In the absence of such conditions, investigation of anaemia in an older adult should mirror that in younger adults. In the very elderly patient, the particular focus of investigation should be on potentially reversible causes.

Infections

Viral infections

SARS-CoV-2

SARS-CoV-2 infection causes a wide variety of pathology in humans with varying chronicity and severity. Infection may be entirely asymptomatic in some, but fatal in others. It may be an acute and self-limiting illness, but in other patients, symptoms that developed during the initial illness persist beyond 90 days, resulting in post-COVID-19 syndrome which can cause significant long-term physical and psychological morbidity. It typically affects the chest, causing pneumonitis, cough and hypoxia, but can present exclusively with symptoms in other organ systems, for example gastro-intestinal. It increases the tendency for thrombosis, especially in the pulmonary vasculature, and increasing prothrombin and D-dimer levels are a marker of disease severity. Rates of haemorrhage, although less common than thrombosis, are also increased with this disease.

The effects of SARS-CoV-2 on the blood count correlate with disease severity, and are of prognostic significance. During acute infection, the severity of thrombocytopenia and lymphopenia correlate with disease severity, likelihood of admission to intensive care unit and death.

Red cells

Both anaemia, and a positive IgG direct antiglobulin test are common in patients with COVID-19, who may have elevations in lactate dehydrogenase and bilirubin, but treatment specifically directed at AIHA is not usually required.

Patients who are hospitalised with COVID-19 are likely to develop anaemia of chronic disease, but attention should be also paid to the correction of haematinic deficiencies, and to the support of renal dysfunction.

White cells

Lymphopenia is a frequent finding, seen in the majority of patients hospitalised with this infection. A progressive decline in lymphocyte count has been described in non-survivors of COVID-19, compared to survivors. Neutrophilia is common in hospitalised patients, especially if dexamethasone is administered. In patients not taking exogenous steroids, the possibility of bacterial co-infection should be considered.

Platelets

Cases of immune thrombocytopenic purpura have been described following infection with SARS-CoV-2, although this does not appear to be common.

Severe COVID-19 is characterised by widespread immune activation, with similarities to haematological conditions such as macrophage activation syndrome, HLH and DIC. The platelet count often falls with increasing disease severity.

Vaccination against SARS-CoV-2

Vaccine-induced Immune Thrombotic Thrombocytopenia (VITT) may occur very rarely in individuals following vaccination with adenovirus vector-based vaccines. It typically develops 5–10 days after vaccination. Patients with this condition develop activating auto-antibodies to platelet factor 4 (PF4) causing a fall in the platelet count. The same antibodies contribute to the activation of neutrophils, monocytes and endothelial cells which, in combination with the inappropriately activated platelets leads to widespread coagulation. Patients develop a thrombotic syndrome which includes typical sites, such as pulmonary embolism and less often deep vein thrombosis of the legs, but also an increased tendency to affect the cerebral, splanchnic and adrenal veins. Less commonly these patients may develop arterial thromboses. Management of this life-threatening condition should be guided from a specialist centre familiar with the management of pro-thrombotic syndromes.

HIV

HIV infection may lead to cytopenias through a number of causes (Table 56.11), including direct infection of the haematopoietic stem cells, or through secondary mechanisms including co-infection by other pathogens, aberrant immune responses, development of malignancies, or the toxic effects of medications used in the management of patients with HIV. Cytopenias are less commonly seen in the modern era of HIV management, however, due to earlier diagnosis, better disease control, and due to advances in combination anti-retroviral treatments which are less cytotoxic than older treatments.

Table 56.11 Mechanisms of development of cytopenia in patients with HIV.

Mechanism	Notes
HIV infection	Through direct marrow suppression, and induction of apoptosis. Anaemia of chronic disease is common.
Co-infection with other pathogens	
• Viral (Hepatitis A/B/C, CMV, EBV)	May be worsened by development of hypersplenism
• Viral (Parvovirus B19)	Causes an isolated anaemia with reticulocytopenia
• Viral (HHV8)	Associated with lymphadenopathy
• Bacterial/fungal/mycobacterial	Through cytokine-induced marrow suppression, or direct marrow infection (for example mycobacterium avium)
• Protozoal	Depending on travel history, consideration should be given to exclusion of malaria (typically presents with anaemia), and visceral leishmaniasis, which presents with pancytopenia and hepatosplenomegaly.
Auto-immune cytopenia	May affect platelets, red cells or neutrophils
Haemophagocytic lymphohistiocytosis	HIV infection can be the trigger for the development of HLH, which is characterised by cytopenias, unexplained fevers and grossly elevated ferritin levels. Haemophagocytosis may be seen in the bone marrow.
Microangiopathic haemolytic anaemia	This may be directly triggered by the HIV infection, or by medications used in its management. Fragments (schistocytes) will be seen on the peripheral blood smear, and the patient may have neurological symptoms.
Malignancy	
• Lymphoma (Hodgkin or non-Hodgkin), including CLL	Cytopenias arise through auto-immune destruction, or anaemia of chronic disease
• Leukaemia	Cytopenias usually through direct marrow infiltration
• Kaposi's sarcoma	This is associated with HHV8 co-infection, and may cause anaemia and thrombocytopenia
Haematinic deficiency	This is common at diagnosis of HIV, due to poor nutritional intake, and gastrointestinal disease involvement. It may contribute to cytopenias

Cytopenias arising directly through HIV infection

Patients presenting with new HIV infections may be noted to have cytopenias, most often lymphopenia (with reduced CD4 count) or neutropenia. Anaemia and/or thrombocytopenia are less often seen at presentation but become more common in patients presenting at later stages of infection, and are frequent in patients whose disease meets the definition for AIDS. HIV infects the haematopoietic stem cells themselves, and inhibits their proliferation, as well as through the production of pro-inflammatory cytokines (e.g. IFN-γ, TNF-α) that induce apoptosis.

Cytopenias arising through co-infection with other pathogens

In individuals with newly detected HIV infection and low CD4 counts, co-infection with other pathogens is commonly detected, and a full screening panel should be performed. Bacterial or fungal infections may contribute to cytopenias. Evidence of chronic parvovirus (erythrovirus) B19 should be sought if there is marked anaemia (with reticulocytopenia), CMV in patients with colitis or retinitis, EBV in those with symptoms resembling infectious mononucleosis, and EBV and CMV in patients with HLH. Human herpes virus 8 may also complicate HIV infection, causing multicentric Castleman disease, characterised by lymphadenopathy, hepatosplenomegaly and cytopenias.

Viral hepatitis may co-infect patients with HIV, and if chronic liver disease and hypersplenism have developed, could contribute to cytopenias.

Cytopenias arising through immune dysregulation

HIV infection may present with auto-immune cytopenias, such as immune thrombocytopenia, AIHA and auto-immune neutropenia, and HIV serology should always be assessed in patients with unexplained cytopenia.

HIV may trigger the development of haemophagocytic lymphohistiocytosis, and ferritin levels should be measured as a screening test to determine whether this syndrome could be contributing to cytopenias.

MAHAs, such as thrombotic thrombocytopenic purpura can be triggered by HIV infection.

Cytopenias related to the development of malignancy

HIV predisposes patients to the development of malignancies, especially haematological, with lymphomas being the most common. The emergence of malignancy is likely to contribute to cytopenias, either through marrow involvement, hypersplenism or auto-immune destruction.

Other viruses

Anaemia

Haemolytic anaemia due to red cell autoantibody production, usually of the warm type, may occur, although cold antibody syndromes may occur in measles, influenza, infectious mononucleosis and mumps. Paroxysmal cold haemoglobinuria is rare and occurs in children due to Donath–Landsteiner IgG anti-P antibodies. Non-immune micro-angiopathic haemolytic anaemia may be associated with thrombotic thrombocytopenic purpura or disseminated intravascular coagulopathy, which may be the result of viral infection. Pure red cell aplasia occurs with parvovirus (erythrovirus) B19 infection which, in patients with pre-existing conditions causing shortened red cell survival (for example sickle cell disease), may cause a 'transient aplastic crisis'. This virus may also cause erythema infectiosum or fifth disease, in children. It invades and destroys red cell progenitors and the aplasia is terminated when neutralising IgM and IgG antibodies develop. If the virus infects pregnant women, it may cross the placenta and cause miscarriage or hydrops fetalis. Intravenous immunoglobulin has been used for severe cases (e.g. in pregnancy, HIV infection and after stem cell transplantation). Bone marrow aspirate shows characteristic giant erythroblasts.

Pancytopenia occurs in virus-associated bone marrow aplasia, for example with hepatitis viruses. The presence of viruses, either within lymphocytes or on their cell surface, may lead to production of a range of cytokines (including TNF, IFN-α and IFN-γ), which inhibit haemopoietic cell proliferation *in vitro* and *in vivo*. This may cause a substantial reduction in erythropoiesis and is presumably the mechanism underlying neutropenia and lymphopenia in viral infections. In infectious mononucleosis and other viral infections such as viral hepatitis, the virus infects B lymphocytes and the activated lymphocytes seen on the blood film are a reactive population of T cells.

Platelets

Thrombocytopenia may occur due to multiple mechanisms. Children with autoimmune thrombocytopenic purpura frequently give a history of a preceding viral illness, and autoantibody production is well described in infectious mononucleosis, rubella and cytomegalovirus infections. Reduction of bone marrow thrombopoiesis is frequently subclinical, but it is particularly important in virus-associated aplasia and dengue fever. Thrombocytosis can also occur in response to viral infections.

Bacterial, fungal and protozoal infections

Specific microbial infections can be associated with particular effects on the haematological parameters (Table 56.12).

Anaemia

Anaemia of chronic disease can occur in acute infections, overwhelming septicaemia and chronic or suppurative infection. Haemolytic anaemia is less common but can occur through both immune (e.g. cold antibodies with anti-I specificity in *Mycoplasma* infection) and non-immune mechanisms. Direct red cell invasion frequently results in severe haemolysis in infections caused by *Bartonella baciliformis*, with elements of intravascular haemolysis (due to increased red cell fragility) and extravascular haemolysis. *Clostridium perfringens* produces an α-toxin (a lecithinase) and a θ-toxin, and *Staphylococcus aureus* an α-toxin, which act as haemolysins and can cause severe intravascular haemolysis. DIC and MAHA can occur in any severe bacterial, fungal or protozoal infection. HUS is associated with a range of bacterial infections, including *Salmonella*, *Shigella* and *Campylobacter* spp., but most frequently with verotoxin-producing strains of *Escherichia coli*. Hookworm and schistosomiasis are two of the most frequent causes worldwide of iron deficiency anaemia, due to intestinal haemorrhage and in the case of schistosomiasis, haematuria (see Chapter 11).

White cells

Neutrophilia is the most common manifestation. The neutrophil response shows great individual variation, with no clear relationship to the severity of the infection. The term *leukaemoid reaction* is used to describe marked leucocytosis ($>50 \times 10^9$/L), with circulating immature forms occurring in patients with non-leukaemic conditions, typically severe infection or haemolysis or with generalised malignancy. Such reactions are more common in children. Features that distinguish such a reactive leucocytosis from chronic myeloid leukaemia include the presence of toxic granulation, Döhle bodies, and the lack of twin peaks of neutrophils and myelocytes. Neutropenia can also occur with virtually any bacterial infection, although it has been most frequently noted with *Salmonella*, *Rickettsia* and *Brucella*. Defects of neutrophil function may also occur.

Platelets

Thrombocytosis is frequent in patients with chronic infections, especially bacterial abscesses, and during the convalescent phase of acute infections. Thrombocytopenia also occurs during severe bacterial or fungal infection, particularly where there is bloodstream invasion or in intensive care patients. Certain rickettsial infections (e.g. Rocky Mountain spotted fever) are almost always associated with thrombocytopenia. Accelerated platelet

Table 56.12 Haematological changes in bacterial, fungal and protozoal infections.

Anaemia
Anaemia of chronic disease
Haemolytic
Immune: *Mycoplasma*, malaria, syphilis (paroxysmal cold haemoglobinuria), listeriosis
Non-immune: *Clostridium perfringens* (toxin-related), *Bartonella bacilliformis* (Oroya fever)
Malaria/trypanosomiasis with microangiopathy/Disseminated intravascular coagulation, septicaemia
HUS: verotoxin-producing *Escherichia coli* and *Streptococcus pneumoniae*
Sequestration
Splenomegaly (e.g. malaria, schistosomiasis)
Blood loss
Helicobacter pylori, *Ancylostoma (hookworm), schistosomiasis*
White cells
Neutrophilia
Virtually any bacterial/fungal infection
Neutropenia
Salmonella, *Rickettsia*, brucellosis, pertussis, disseminated tuberculosis (TB)
Overwhelming septicaemia
Lymphocytosis
Whooping cough (*Bordetella pertussis*), *Rickettsia*
Lymphopenia
TB, acute bacterial infections, brucellosis, acute viral infections, including *SARS-CoV-2*
Eosinophilia
Aspergillosis, coccidioidomycosis, *Chlamydia*, streptococcal infections, *Ancylostoma*
Eosinopenia
Common in acute *Bacteroides* infections
Monocytosis
Subacute/chronic infections (e.g. disseminated TB, listeriosis)
Pancytopenia
Bone marrow suppression (e.g. disseminated TB, listeriosis)
Haemophagocytosis: septicaemia
Peripheral destruction (e.g. DIC)

destruction is the most frequent mechanism and can arise through DIC or microangiopathy with platelet attachment to damaged endothelium. Immune destruction can also occur. Decreased platelet production is a less common mechanism, but may occur (e.g. in disseminated tuberculosis). The inflammatory and procoagulant responses to infections are related. TNF-α, IL-1α and IL-6 may activate coagulation and inhibit fibrinolysis, whereas thrombin may stimulate inflammatory pathways. In severe infection, the end result may be endovascular injury, DIC, multiorgan failure and death.

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CHAPTER 57

Consultative haemostasis and thrombosis

57

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Introduction

Most of the information set out in a standard textbook such as this is organised in functional or anatomical systems. This is logical but does not necessarily represent the way problems present themselves in practice. Haematologists specialising in thrombosis and haemostasis will have primary responsibility for a wide group of patients often with hereditary or lifelong disorders but much of their time is spent giving advice in consultation regarding the management of patients with other primary disorders but who have developed bleeding or thrombotic complications or whose history and therapy for these problems complicates their management. Trainee haematologists frequently comment on the high proportion of their calls arising from consultative haemostasis and thrombosis. This chapter is an attempt to give some structure to managing these problems. Some of the information will appear in other chapters but is here organised in a practical way that reflects how problems arise in practice and often complicate each other.

The bleeding patient

The post-operative patient

Surgery is necessarily traumatic, although in many cases much less so than in the past. However, the advent of laparoscopic or keyhole surgery has made it more difficult to detect bleeding when it occurs. The time taken for haemodilution means that a fall in haemoglobin may take 12–24 hours to appear after a bleeding event and so tachycardia and (postural) hypotension should be sought as they can be significant early indicators of blood loss. Major haemorrhage definitions are not useful in a clinical time frame, but significant bleeding (>150 mL/min) without immediate prospect of control or heart rate >110 beats/min and/or systolic blood pressure <90 mm Hg are useful indicators. Drugs, patient age and comorbidity may make these measures more difficult to assess. The contents of surgical drains should also be examined. When visible, the pattern of bleeding may be helpful in diagnosis and management. Local bleeding or bleeding via drains may be anatomical or surgical whereas widespread bleeding from non-surgical sites indicates deranged haemostasis. Widespread ecchymoses and bleeding from intravenous lines may indicate disseminated intravascular coagulation (DIC), purpura may indicate small vessel thrombosis as seen in meningococcal sepsis or pyoderma gangrenosum. Large unstable and friable clots are typical of haemophilia. Delayed bleeding, whilst theoretically typical of fibrinolysis, reactive haemorrhage or infection may actually arise from almost any haemostatic defect.

Routine preoperative coagulation testing is not recommended due to its poor predictive power, but if previous coagulation screens and platelet counts are available, they can be valuable by excluding the presence of a significant hereditary coagulation disorder and identifying any current abnormalities as acquired. This limits the differential diagnosis considerably.

Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).
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Nonetheless, it is not uncommon for an abnormal coagulation screen disclosing undiagnosed von Willebrand disease (VWD) or mild haemophilia to have been overlooked in preoperative assessment and mild coagulation factor deficiencies may not cause prolongation of the standard prothrombin time (PT) and activated partial thromboplastin time (APTT) screening tests. Thrombocytopenia due to an underlying disorder will usually have been detected preoperatively and its development post operatively is likely to indicate consumption.

It is important first to obtain a clear picture of the amount and rapidity of blood loss followed by the amount and type of blood products that have been given. Coagulation tests and a full blood count (FBC) should be sent but will take time to become available and the situation may be urgent. Increasingly, the results of near-patient testing such as thromboelastography (TEG) or rotational thromboelastometry (ROTEM) will be available. The global nature of these viscoelastic tests is useful in the context of acute bleeding and if they are normal then post-operative or traumatic bleeding is unlikely to be due to a defect of haemostasis and more likely to be structural.

The basic viscoelastic profile is shown in Figure 57.1. The initial clotting time (R or CT) reflects the time taken for thrombin generation to increase and is typically prolonged by anticoagulants, inhibitors or coagulation factor deficiency, including fibrinogen. The speed with which the clot then forms can be measured as the rate or the angle (α-angle) at which the trace widens. This is also dependent on thrombin generation but requires fibrinogen and platelets as well. The maximum amplitude or clot firmness is dependent on both fibrinogen and platelet function. Finally, if fibrinolysis is excessively active, the breakdown of the clot will be evident as decrease in the width of the trace. Normal fibrinolysis usually takes too long to be routinely observed. Abnormalities of the viscoelastic trace can therefore be used to generate straightforward algorithms to help guide replacement therapy. An example is shown in Figure 57.1. This approach is approved by NICE for cardiopulmonary bypass (CPB) surgery but it is widely used in other circumstances as well. In general, haematologists are less familiar with these tests but a working knowledge of their interpretation is invaluable for consultative work. Nonetheless, they do not determine the aetiology of any abnormality which requires further investigation with specific laboratory tests.

Viscoelastic devices are in evolution and new ultrasonic methods for detecting clot strength are replacing physical ones and multitest cartridges are being used to avoid errors from pipetting. Moreover, new reagents allow dissection of platelet-fibrinogen, anticoagulant and antiplatelet effects (platelet mapping).

Dilutional coagulopathy

In bleeding patients, resuscitation is usually focussed, quite reasonably, first on simple fluids and then on red blood cells (RBC) to maintain oxygen delivery. However, RBC preparations do not contain any plasma components and so without plasma replacement, dilutional coagulopathy is inevitable. If no coagulation tests are available, then the likelihood of dilutional coagulopathy can be reasonably estimated by counting the number of units of RBC transfused compared to the number of units of plasma, cryoprecipitate and platelets. If more than about 6 units of RBC have been given with 3 units fresh frozen plasma (FFP) or less, then dilution is likely. Remember that in dilutional coagulopathy fibrinogen falls early and platelets late, whilst the reverse is true of DIC. Routine coagulation tests confirm dilutional coagulopathy likely to cause bleeding when the PT ratio is >1.5 and fibrinogen <0.8 g/L. Viscoelastic tests will give similar indications and can be used to direct replacement as above, but dilutional coagulopathy can be prevented by beginning plasma replacement alongside red cell transfusion as soon as it is recognised that the haemorrhage may be large. This realisation has led to development of major haemorrhage protocols which provide RBC, plasma and platelets in fixed proportions from the beginning of resuscitation. A major haemorrhage protocol is now in place in most major hospitals and a suggested example is shown in Figure 57.2.

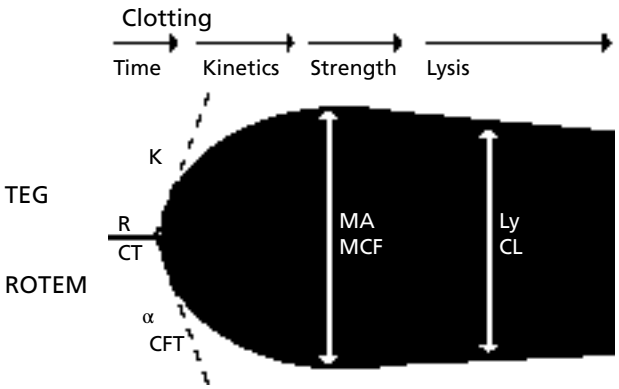
If dilutional coagulopathy is established and rapid replacement required or volume capacity is limited, then use of concentrates may be considered. A combination of prothrombin complex concentrate (PCC), fibrinogen concentrate or cryoprecipitate and platelets will provide an almost complete haemostatic mechanism (factor VIII and von Willebrand factor (VWF) release from endothelial stores mean that they are rarely depleted). Current guidelines note that evidence for routine use of this approach is limited and also caution against the use of these concentrates as monotherapy for obvious reasons. Thus, in an emergency, concentrates and platelets should be given simultaneously or in rapid succession, since they must all be present at the same time for successful haemostasis. Recombinant factor VIIa (FVIIa) has been used as an adjunct to promote haemostasis and although generally not approved for this purpose it has now been authorised for refractory post-partum haemorrhage.

The trauma patient

The coagulopathy associated with Trauma is dealt with in Chapter 43 'Acquired coagulation disorders'.

The post-partum patient

Parturition is a highly haemorrhagic event. Recent studies have established that the physiological rises in factor VII, factor VIII and fibrinogen during pregnancy are important



	Clot initiation	Clot strength		Clot lysis
Test result shows	Prolonged R, ACT or CT	Reduced MA/MCF, normal fibrinogen†	Reduced MA/MCF, low fibrinogen†	LY-30 >8% Li30 >15%
What does this mean?	Low clotting factors and/or low fibrinogen level Warfarin use Heparin use DOAC use (not Apixaban)	Low platelets	Low fibrinogen	VHA detected fibrinolysis*
Therapy recommended	FFP (PCC might be considered)	Platelets	Cryoprecipitate or fibrinogen concentrate	Consider additional anti-fibrinolytic*
Therapy groups				
Obstetric	FFP if R or CT above the normal range. PCC is not recommended	No data are available to guide platelet transfusion	Cryoprecipitate or fibrinogen concentrate if FIBTEM <7 mm or <12 mm in severe bleeding	No data are available for guiding antifibrinolytic therapy
Liver	FFP if results above normal range (PCC might be considered)	EXTEM MCF <35 mm	FIBTEM <7 mm	Fibrinolysis at reperfusion may correct spontaneously. Anti-fibrinolytics are indicated in most other circumstances.
Cardiac	FFP if>15% above ULN	Platelets	Cryoprecipitate or FgC	
Trauma	FFP if results ≥ULN PCC not recommended	Give platelets if MA/MCF at lower end or below the normal range whilst ff or FIBTEM normal	Cryoprecipitate or FgC if FIBTEM or ff at lower end or below the normal range	TEG LY-30 ≥3% indicates clinically important lysis

The research in the top panel was originally published in *Blood*. Johansson *et al* (2014) © the American Society of Hematology.
ACT, activated clotting time, CT, clotting time, DOAC, direct oral anticoagulant, ff, functional fibrinogen; FFP, fresh frozen plasma; FgC, fibrinogen concentrate; Li30, lysis index at 30 min; LY-30, lysis at 30 min; MA, maximal amplitude, MCF, maximum clot firmness; PCC, prothrombin complex concentrate, R, reaction time; TEG, thromboelastography; ULN, upper limit of normal; VHA, viscoelastic haemostatic assay.

*Do not withhold an anti-fibrinolytic if is it clinically indicated and within 3 h of injury or start of postpartum haemorrhage.
†Clot strength measures are highly dependent on platelets and fibrinogen. To differentiate low clot strength due to loss of platelet function or from that due to low fibrinogen levels, the clot strength of the fibrinogen assay (ff – TEG; FIBTEM – ROTEM) should be compared with the standard TEG or ROTEM EXTEM trace, respectively. A low overall clot strength with a normal ff/FIBTEM would suggest lack of platelets contributing to the clot, whereas a low overall clot strength with a concomitant low ff/FIBTEM would point to lack of fibrinogen.

Figure 57.1 TEG and ROTEM traces. Thromboelastography (TEG) and thromboelastometry (ROTEM) traces can be broadly divided into four main sections: (a) time to first fibrin formation [reaction *time – TEG5000; activated clotting time (ACT) – TEG6s; clotting time (CT) – ROTEM]; (b) kinetics of fibrin polymerisation which has two parameters that are directly interchangeable [alpha angle used by both devices) and additionally, the time it takes from first fibrin formation to when arbitrarily the clot is 20 mm wide (kinetics [K] time – TEG5000; clot formation time [CFT] – ROTEM); (c) measures of clot strength – often demarcated at 5 min intervals (amplitude [A] – A30 – TEG clot strength at 30 min; clot amplitude [CA] – CA30 – ROTEM clot strength at 30 min) and with a peak measurement (maximal amplitude [MA] – TEG; maximum clot formation [MCF] – ROTEM); (d) measures of clot breakdown/lysis, usually presented as a percentage of reduction in the clot strength measure compared to maximal measurement)Lysis (Ly) – TEG; clot lysis/lysis index/maximal lysis (CL/Li/ML) – ROTEM) (Source: Reproduced from: Curry *et al*. 2018/With permission of John Wiley & Sons.)

for peri-partum haemostasis and that non-pregnant normal ranges are not adequate. The major cause of post-partum haemorrhage is uterine atony as well as other placenta-uterine abnormalities. Hence the initial management is largely obstetric but with rapid resuscitation, dilution can easily occur and coagulopathy may be complicated by problems such as amniotic fluid embolism, thrombocytopenia (gestational, pre-eclampsia (PET), haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome), sepsis, abruption. As with other major haemorrhages, fluids should be warmed if possible

Figure 57.2 **Algorithm for the management of major haemorrhage (adapted from the BCSH *Practical Guideline for the Management of Those With, or At Risk of Major Haemorrhage* (2013) with permission)**

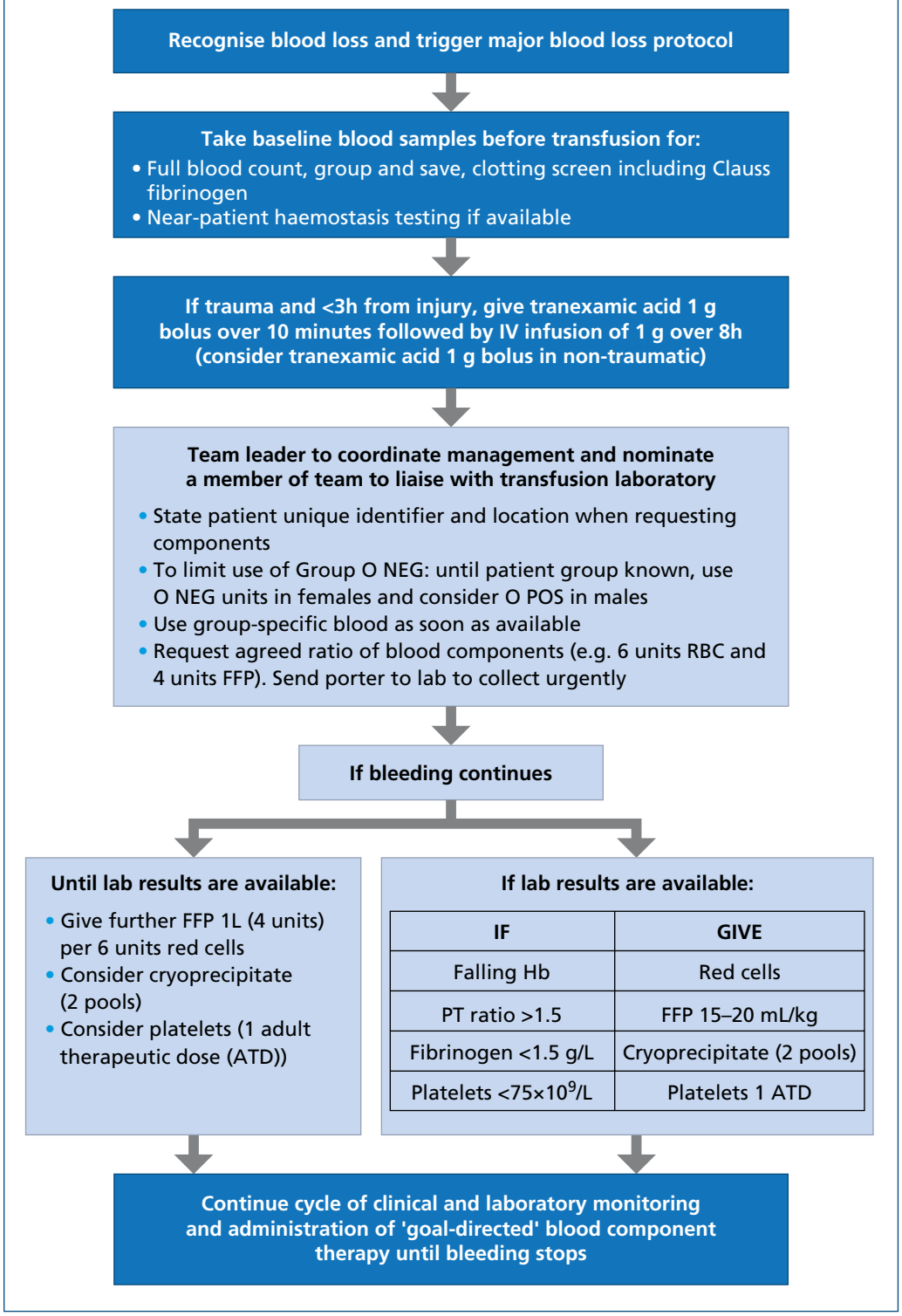


Figure 57.2 Major haemorrhage protocol (Source: Derek Norfolk 2013/TSO/Public Domain OGL.)

to avoid hypothermia and acidosis. Suggested targets (for ROTEM) are

- Normal PT/aPTT (refer to local laboratory ranges)
- Fibrinogen (>2 g/L)
- EXTEM CT (<75 s)
- Fibtem A5 (>11 mm)

Pregnancy-related haemostatic dysfunction is dealt with in more detail in Chapter 43 'Acquired coagulation disorders'.

The patient bleeding with normal coagulation tests

Sometimes post-operative patients appear to have been given adequate replacement therapy and have corrected their standard coagulation tests but continue to bleed or they have bled despite normal tests. Obviously, the first consideration is whether this is structural or surgical and a normal TEG/ROTEM makes this likely. The unrecognised presence of antithrombotic therapy should be considered because anticoagulants, even at therapeutic doses, do not always produce an abnormal coagulation screen or viscoelastic test and nor do anti-platelet agents. Consider that the available analyses may not have tested all haemostatic functions such as platelet capture under shear stress, factor XIII activity, anticoagulant function and fibrinolysis. Some procedures such as prolonged CPB or hypothermic arrest procedures typically result in a platelet dysfunction due to loss of surface glycoproteins not detected by standard tests. Extravascular circuits can cause an acquired von Willebrand syndrome which can be associated with angiodysplasia and refractory bleeding and the factor VIII may not be reduced sufficiently to prolong the APTT.

Additional non-replacement therapies for haemorrhage

Tranexamic acid is a lysine analogue that binds to the lysine binding sites on plasminogen to prevent it from binding to fibrin and being activated by tissue plasminogen activator (tPA). It has been shown to reduce blood loss in major orthopaedic, cardiac and general surgery. Notably in the CRASH II study, early administration within three hours, but most markedly within 1 hour, reduced mortality. This has led to a number of other trials that have shown variable success and in some cases harm. Meta analyses suggest that there is overall no increase in thrombosis associated with tranexamic acid use but this has been increased in some studies. High doses in particular can increase the risk of seizures. The trial results are summarised in Table 57.1.

Aprotonin is a bovine serine protease inhibitor used as a potent anti-fibrinolytic although some of its beneficial effects may result from other effects including inhibition of thrombin platelet activation. After reports of an increase in myocardial infarction and death, it now has a limited indication for cardiac surgery patients at high risk of bleeding.

Desmopressin is a modified form of vasopressin without its pressor effect, that releases stores of von Willebrand factor and Factor VIII from endothelial stores. It is widely used to elevate levels of these factors in suitable patients with forms of haemophilia and VWD. It is also effective in mild platelet function defects and in reversing the primary haemostatic defect in uraemia, but has not been shown to be of general benefit in haemorrhage.

The outpatient with bleeding symptoms

Acute onset bleeding

Sometimes patients present with new-onset bleeding symptoms as the primary problem. Once anticoagulant excess has been excluded, the differential diagnosis in adults is relatively short. A full blood count will demonstrate whether acquired disorders such as immune thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP) or leukaemia are the explanation and the clinical picture of thrombocytopenic bleeding will be typical. Widespread ecchymoses and

Table 57.1 Trial results for tranexamic acid.

Indication	Benefit on blood loss/mortality	Thrombotic risk reported	Additional risk	Comment
Trauma	Yes	No		Timing important
TBI	Yes	No		
ICH	No	No		
GI	No	Yes	Seizures increased	4 g/24 hrs
Post-partum	Yes	No		Timing important
Orthopaedic surgery	Yes	No		
Cardiac surgery	Yes	No	Seizures increased	
HMB	Yes	Possible, but causality uncertain.		

soft tissue bleeding raises the possibility of acquired haemophilia supported by a newly prolonged APTT and confirmed by a low factor VIII level. Typical patients are elderly, sometimes with comorbidity, or younger and post-partum. Acquired deficiencies of other factors can occur but are extremely rare: Factor V and factor XIII are probably the most common and the problem should be evident from coagulation tests of PT and APTT and specific assays.

Acquired von Willebrand syndrome is often asymptomatic and detected on laboratory testing but may present with protracted gum or mucosal bleeding and bruising after minor trauma. A number of disorders including paraproteinemia, aortic stenosis and myeloproliferative neoplasms (with thrombocytosis) can be responsible, each via different mechanisms. There is a well-known association between chronic blood loss from angiodysplasia and acquired von Willebrand syndrome secondary to aortic stenosis. Note that there may be acquired FVIII deficiency but no anti-FVIII antibody or inhibitor.

Some tumours, notably prostatic carcinoma, release excessive profibrinolytic factors (urokinase-plasminogen activator (uPA) or tPA) into the circulation causing a haemorrhagic disorder due to primary fibrinogenolysis. Clinical bleeding from gut or urogenital tract may result but also large subcutaneous or muscular haematomas reminiscent of acquired haemophilia. Blood tests show the expected reduction in fibrinogen, gross elevation of fibrin degradation products (FDPs) and prolongation of clotting times. D-dimer levels are also elevated suggesting there is also a component of DIC.

Bruising

Bruising, often reported to occur with minimal or no trauma, is one of the commonest reasons for referral to the haemostasis clinic. It is usually very clearly distinct from the large soft tissue and subcutaneous ecchymoses seen in acquired haemophilia and also from the large patchy lesions of senile purpura over the back of the hands and forearm in association with delicate sun-exposed skin or prolonged steroid therapy. Easy bruising characterised by small bruises predominantly on the legs and sometimes arms but not the torso is very rarely an indicator of a significant underlying bleeding disorder.

In older patients, the appearance of bruises may follow initiation of antiplatelet therapy, steroid inhalers or over-the-counter (OTC) supplements with anti-platelet activity. Selective serotonin reuptake inhibitors (SSRIs) are widely used and have a clear antiplatelet effect manifest as an increased risk of intracranial haemorrhage but bruising or other bleeding may also be enhanced. A history of surgery without problems is helpful. Some patient groups will be at risk of scurvy and older patients with amyloidosis may present with bruising. Routine investigations are usually warranted as the acquired disorders listed above are significant and others such as paraproteins or myelodysplasia may also be associated with impaired platelet function. Nonetheless, most instances of 'easy bruising' especially when restricted to the limbs do not reflect a significant haemostatic defect and preliminary investigations are normal with no need for further testing. Bruising is a feature of some forms of Ehlers-Danlos syndrome but not of Marfan's or cutis laxa. Bruises preceded by a painful sensation have been described as 'painful bruising syndrome'. The aetiology is not clear but may represent inadvertent self-infliction.

Post-viral bruising in children may be caused by ITP but there is also a syndrome of bruising and prolonged coagulation tests produced by acute but transient antiphospholipid antibodies. It does not require treatment.

Epistaxes

Epistaxes are common in childhood and usually settle in adolescence; this pattern is not indicative of a bleeding disorder. Indicators of a bleeding disorder are prolonged bleeding, usually 15–20 min or more, recurrence after local measures and bleeding from both nostrils. An ENT assessment will be useful at some stage and although cautery may be beneficial, repeated treatments may be counterproductive especially if the underlying problem is haemostatic. Prolonged epistaxes are a typical feature of VWD and platelet disorders, but also remember to check for the telangiectasia of hereditary haemorrhagic telangiectasia (HHT), which is a progressive disorder and so lesions may be less obvious in young patients.

Heavy menstrual bleeding

There is a growing realisation that the problem and impact of heavy menstrual bleeding (HMB) has been underestimated if not ignored, by haematologists and physicians in general. It is highly prevalent and an estimated 30% of women report HMB at some point in their reproductive period and 15–30% of these may have an underlying bleeding disorder. It should always be taken seriously and investigated for both haematological and gynaecological contributory factors. Pictorial assessment tools are available to give quantitative assessment if necessary and Bleeding Assessment Tools, developed primarily for VWD may indicate help the identification of a likely bleeding disorder. Heavy bleeding from menarche and the development of iron deficiency are indicators of severity.

Close collaboration between haematology and gynaecology is essential, firstly because although specific treatments are available for haemostatic support, the first line of therapy is usually hormonal. Combined or progesterone-only contraceptive pills, the mirena coil or loop are all valuable methods for reducing menstrual blood loss. Tranexamic acid may be useful on its own in mild cases or as an adjunct. Severe bleeding disorders such as type 3 VWD or if contraception is unwanted may necessitate intermittent replacement therapy. Secondly, HMB may have a pathological or structural basis requiring gynaecological management. The

National Institute for Health and Care Excellence (NICE) has published guideline https://www.nice.org.uk/guidance/ng88.

Bruising in children

Hereditary disorders of haemostasis generally present at a young age, often with bruising, haemarthrosis, epistaxis or other post-traumatic bleeds which are spontaneous or out of proportion to injury. Careful history-taking and critical examination are essential. Many children with haemophilia have been taken into care erroneously, but it remains important to consider deliberate trauma and to activate safeguarding authorities when appropriate.

The patient with abnormal coagulation tests

The coagulation screen

The coagulation 'screen' routinely requested in hospital almost always comprises a PT and an APTT. In some hospitals, a fibrinogen assay is included or this may be a reflex test only performed if one of the first two is abnormal. Coagulation testing is often performed without any clear purpose and consequently, it frequently reveals abnormal results of uncertain significance resulting in a call to haematology for advice. At the same time, a normal coagulation screen may give unwarranted reassurance that haemostasis is normal. The PT and APTT bear only limited relationship to the *in vivo* process of haemostasis and do not require the elements of primary haemostasis (platelets, VWF and collagen). Nor do they assess clot stability, anticoagulant mechanisms or fibrinolysis. Conversely, they may frequently be rendered abnormal by factors that have no impact on haemostasis such as factor XII deficiency or a lupus anticoagulant. The most important lesson for the haematologist is that a normal coagulation screen does not imply normal haemostasis and an abnormal one does not indicate abnormal haemostasis. It is essential to understand both the limitations and the importance of these tests.

When approaching the patient in hospital with abnormal test(s) some basic steps are always useful:

1 Has the patient ever had normal coagulation tests? If so then this excludes hereditary or congenital coagulation disorders and narrows the differential diagnosis considerably.

2 Has the patient ever had interventional procedures or trauma and were these associated with bleeding? This similarly can help exclude or identify a congenital disorder.

3 Could the abnormality be artefactual due to anticoagulants (including heparin from the sample line or cannula), under filling of the sample tube, elevated haematocrit, prolonged transit or is it a failure of endpoint detection by the analyser rather than a genuine prolongation? If the endpoint was not detected, perform a manual test or inspect the waveform on the analyser. In some laboratory systems, the plasma is separated before shipping to another site for analysis so that an incorrect sample, for example in EDTA, may have been sent.

4 A repeat test with careful collection and transport is always valuable.

5 What is the sensitivity of your APTT reagent to nonspecific/antiphospholipid antibodies?

6 What type of endpoint detection does your analyser use? Optical end-point detection may be affected by other plasma factors such as lipids or haemolysis.

7 Does the patient have liver dysfunction, malabsorption or a prolonged reduction in dietary intake? In which case an injection of vitamin K may improve results significantly within 12–24 hours.

Isolated prolonged PT

This indicates Factor VII deficiency or an inhibitor. Some PT reagents are sensitive to antiphospholipid antibodies. It may also be seen at the beginning of VKA therapy or incipient vitamin K deficiency. A factor VII assay is required, but unless very long it is unlikely to represent a significant bleeding risk because only very low levels of factor VII (<10 iu/dL) are required for normal haemostasis. If the thromboplastin used is animal rather than human then repeat using one containing human tissue factor because rare variants (factor VII Padua) give abnormal results with animal tissue factor.

Isolated prolonged APTT

This is the most frequent problem in hospital. If the situation is urgent then the best diagnostic approach is to perform factor VIII, IX and XI assays. If these are normal then there is unlikely to be a significant bleeding tendency and surgery can proceed. Other explanations such as a lupus anticoagulant or contact factor deficiency can be investigated later.

Mixing tests are often requested but have limited sensitivity and specificity, use up valuable time and sample and are not recommended in the acute setting providing assays are available. The rationale for this is as follows:

> If mixing tests correct you have not excluded a time-dependent inhibitor and if it is a factor deficiency you do not know which one is missing, so assays will be required.
> If the mix fails to correct you will need to exclude acquired haemophilia by performing a factor VIII assay (or rarely some other specific inhibitor).
> The degree of correction is often difficult to assess especially with minor degrees of prolongation.

If there is insufficient time to perform assays, then consideration of history, tests and current clinical situation may allow you to conclude that a significant problem is unlikely. An extremely long APTT makes a contact factor deficiency or heparin contamination

likely. If the analyser fails to detect an endpoint (e.g. reports >180 s) then perform a manual test: the result may well be normal.

A new and significant (not borderline) isolated APTT prolongation raises the possibility of acquired haemophilia which is perhaps the single most significant and important possibility. A factor VIII assay is required to exclude this. A UK study reported that 33% of patients with acquired haemophilia A (AHA) did not require haemostatic treatment so bleeding may not be evident at diagnosis.

Prolonged APTT and PT

Some judgement is required to conclude that this is a combined problem and not (for example) a significantly prolonged APTT with a marginal prolonged PT which might be better treated as an isolated prolonged APTT. If the abnormality is long standing, perhaps with a bleeding history then assays of factor V, X and II will be indicated and review of the fibrinogen level. If the factor V is reduced, remember that this may be part of the autosomal recessive combined factor V and factor VIII disorder. Other combined deficiencies are extremely rare (see Chapter 42).

Acquired prolongation of both tests may indicate liver impairment, DIC, dilution, vitamin K deficiency and drug effects. The need for correction is complicated and depends on the likely cause but it is not usually required unless bleeding is present or surgery planned. These causes are discussed further in the chapter on acquired disorders. (Chapter 43)

The patient on anticoagulants, antiplatelet agents or following fibrinolytic therapy

The initial step in considering a patient who is bleeding on antithrombotic therapy is to assess the severity of bleeding against the background thrombotic risk, which may entail multidisciplinary discussion, and to obtain details of drug administration including the route, timing and dose, and the presence of any relevant co-morbidities including background predisposition to bleeding and recent invasive procedures or trauma. A suitably sited and maintained point-of-care coagulometer in the Emergency Department reduces delays in correction of warfarin-associated coagulopathies. The major haemorrhage protocol should be activated if necessary.

On top of the immediate management, consideration should be given to the underlying cause of the bleeding in addition to antithrombotic therapy and to the co-administration of any medication associated with a pharmacokinetic or pharmacodynamic interaction. Blood samples should be sent to check the FBC, clotting screen, renal and liver function tests and, where available and appropriate, an anti-Xa or drug level. Local measures to control bleeding should be implemented as appropriate.

Heparin

The risk of major bleeding during the initial days of therapeutic heparin administration is about 1–5%. Intravenous unfractionated heparin (UFH) has a short half-life of about 60–90 min, unless administered in considerable excess and it may be sufficient to simply switch off the infusion in the event of minor bleeding. More rapid reversal is achieved by the intravenous administration of protamine sulfate which completely neutralises UFH. Subcutaneous low-molecular-weight heparin (LMWH) has a longer effective half-life of 4–6 hours and, although the anti-IIa component is neutralised by protamine sulfate (normalising the APTT), the predominant anti-Xa component is only partially neutralised.

In the event of major bleeding or risk of bleeding, intravenous protamine sulfate should be administered at a rate not exceeding 5 mg/min and with a maximum dose of 50 mg. As a general rule for reversal of UFH being given by intravenous infusion, 1 mg protamine sulfate should be given for every 100 IU heparin administered in the previous 90 min plus 0.5 mg for every 100 IU administered in the previous 90–180 min. For reversal following an intravenous UFH bolus administered less than 30 min earlier, 1 mg protamine per 100 IU UFH should be given. This dose of protamine should be halved if 30–120 min have elapsed since the UFH bolus and a 25% dose should be considered if 120–180 min have elapsed since the UFH bolus. The half-life of protamine sulfate is short (about 7 min) and the APTT should be re-checked 10 min, 2 hours and 6–8 hours after administration, initially to assess reversal and later to identify possible heparin rebound.

For reversal of LMWH, 1 mg protamine sulfate should be administered per 100 IU of tinzaparin or dalteparin or per 1 mg enoxaparin if the heparin has been administered in the previous 8 hours (maximum dose 50 mg). If 8–16 hours have elapsed since the last dose of LMWH, the dose of protamine sulfate should be 0.5 mg per 100 IU of tinzaparin/dalteparin or 1 mg enoxaparin. The APTT should be rechecked after administration if initially prolonged.

Protamine sulfate may cause hypersensitivity including anaphylactoid reactions with bronchospasm and hypotension. Particular care with close monitoring is required in patients who have recently undergone procedures such as cardiopulmonary bypass that use protamine, those with fish allergy or diabetes treated with isophane insulin, or males with a previous vasectomy or male infertility. In excess, protamine sulfate has an anticoagulant effect.

Warfarin and other vitamin K antagonists (VKAs)

The absolute risk of major bleeding on VKAs is about 2% per year overall but is strongly age-related. Major or life-threatening bleeding requires stopping the VKA and administration of PCC

25–50 U/kg and vitamin K 5 mg intravenously. Vitamin K takes several hours to reverse the coagulopathy of VKAs whereas PCC rapidly corrects it but wears off over some hours in line with the half-lives of the vitamin K-dependent clotting factors. PCC in the UK contains the four vitamin K-dependent clotting factors – factors II, VII, IX and X – as well as protein C and protein S. It also contains a small amount of heparin and should therefore be avoided in patients with heparin-induced thrombocytopenia. As well as measurement before administration, the international normalised ratio (INR) should be checked 10–30 min and 4–6 hours after PCC has been given. If PCC is not available, FFP 10–15 mL/kg should be considered but compared to PCC, VKA reversal with FFP is slower and less complete.

Direct oral anticoagulants

The specific agent as well as the timing and amount of the last dose should be established as soon as possible. In the setting of major haemorrhage, if it is less than 2 hours since ingestion, oral activated charcoal 50 g can be considered. Blood samples should be sent for FBC, clotting screen, renal and liver function tests and, if available, the specific direct oral anticoagulant (DOAC) concentration. The latter can be helpful particularly where there is uncertainty over timing of the last dose and given the insensitivity of clotting screens to factor Xa inhibitor concentrations, although a normal thrombin time effectively excludes the presence of dabigatran. A DOAC concentration threshold of 50 ng/mL is reasonable for consideration of reversal in patients with major bleeding, although a threshold of 30 ng/mL can be considered if bleeding is life-threatening. Further anticoagulation should be interrupted until the bleeding has stopped and an assessment made of appropriateness and timing of restarting.

For patients on dabigatran, a specific monoclonal antibody, idarucizumab, is licensed for reversal. The dose is 5 g intravenously given as two consecutive 2.5 g vials over 5–10 min. In an emergency, the dose can be administered over 3–5 min. Usually, the reversal of dabigatran is complete but if bleeding continues or potential re-bleeding would be life-threatening and the APTT or dabigatran level remain elevated, a second 5 g dose can be considered.

Andexanet alfa is a recombinant human factor Xa protein that has been modified to lack both the active serine site of factor Xa and the Gla domain. By giving it in excess over factor Xa, it serves as a decoy protein to which the Xa inhibitor predominantly binds. Andexanet alfa is licensed for life-threatening or uncontrolled bleeding in association with rivaroxaban or apixaban, but NICE approval in England and Wales is restricted to life-threatening or uncontrolled bleeding in the gastrointestinal tract. It has a short half-life and is administered as an initial intravenous bolus followed by a 120-min infusion.

In settings where urgent reversal of an oral factor Xa inhibitor is desired and andexanet alfa is not available or licensed, PCC 25–50 units/kg can be administered but this should not be considered an antidote and is off-label. Activated PCC (factor VIII inhibitor bypassing activity (FEIBA)) 50 units/kg is a less frequently used alternative. Tranexamic acid (1 g bolus over 10 min followed by 1 g infusion over 8 hours) should also be considered.

Other anticoagulant agents

Neither argatroban nor fondaparinux has a reversal agent available and therefore treatment of bleeding on these agents is largely supportive. Limited anecdotal evidence suggests that recombinant factor VIIa (90 μg/kg) might be of some benefit in the setting of life-threatening bleeding on fondaparinux. Argatroban has a short half-life of approximately 60 min but that of fondaparinux is approximately 20 hours and is dependent on renal function.

Antiplatelet agents

The PATCH trial, a randomised trial that investigated the effects of platelet transfusion in patients with antiplatelet therapy-associated spontaneous intracerebral haemorrhage, found that those who received a transfusion had a significantly worse outcome at 3 months than those who did not receive a transfusion. As a result, platelet transfusions are generally not indicated, at least in patients with intracerebral haemorrhage taking aspirin who formed the majority of participants and received a single pool of platelets. Patients taking P2Y12 inhibitors such as clopidogrel, who received 2 pools of platelets, were under-represented in the trial, and those who were for or were more likely to undergo neurosurgical intervention were not included. Pending results from further trials this suggests that patients on P2Y12 inhibitors or undergoing neurosurgical intervention should be managed on an individual patient basis. Patients with intracerebral haemorrhage on aspirin and/or dipyridamole should generally be managed without platelet transfusion unless there is another indication such as thrombocytopenia (platelet count $<100 \times 10^9$/L). Tranexamic acid 1 g bolus followed by 1 g over 8 hours should be considered although it was not of benefit by day 90 in the TICH-2 trial in patients with spontaneous intracerebral haemorrhage, approximately 25% of whom were on antiplatelet therapy at the time of the bleed.

In general, platelet support is not required for extracranial haemorrhage in patients on antiplatelet therapy except as part of the major haemorrhage protocol or if bleeding is life-threatening.

Thrombolytic therapy

Thrombolysis with recombinant tPA (alteplase) for acute stroke is associated with a 2–7% risk of symptomatic intracranial haemorrhage. The half-life of alteplase is about 4 min but,

despite its relative fibrin specificity, it frequently causes a coagulopathy with reduction in plasma fibrinogen and prolongation of the PT and APTT that may last over 24 hours. Symptomatic intracranial haemorrhage attributed to alteplase occurs within 36 hours of the infusion with median times ranging from 5 to 10 hours. Data on management are limited but cryoprecipitate (2 pools) should be administered and the plasma fibrinogen kept above 1.5 g/L. Plasma should be given as a source of antiplasmin and factor V. Tranexamic acid 1 g intravenous bolus followed by an infusion of 1 g over 8 hours also seems reasonable but evidence of benefit is lacking. The outcome is poor with 50% mortality and significant morbidity in those who survive.

Resumption of anticoagulation following major haemorrhage

Multidisciplinary discussion is often required to discuss the benefits, risks and timing of the reintroduction of anticoagulant therapy and possible alternative approaches. Decisions will depend on the indication for anticoagulation, the site and any local cause of bleeding, and the presence of risk factors for further bleeding. Modifiable risk factors, e.g. hypertension or co-administration of antiplatelet therapy, should be corrected where possible. Patient preferences are an important consideration.

For patients with atrial fibrillation, factors that might favour withholding rather than restarting DOACs after a major gastrointestinal (GI) bleed or intracranial haemorrhage ICH on anticoagulants are shown in Table 57.2. Often it is possible to resume anticoagulation 4–7 days after a GI bleed whereas guidelines favour a delay of at least 4 weeks if anticoagulation is resumed after intracranial haemorrhage. The choice of oral anticoagulant agent should be reviewed, DOACs being associated with a lower risk of intracranial haemorrhage than warfarin. Consideration should be given to the potential role of left atrial appendage occlusion when anticoagulant resumption is contra-indicated.

In patients with venous thromboembolism (VTE), the need to resume anticoagulation will be higher in the initial stages of treatment for a recent event than for those continuing for secondary prevention after three months of initial therapy. If major bleeding occurs within the first three months of treatment, anticoagulation is generally resumed as soon as practical after the bleeding has stopped. This is often with LMWH initially because of its shorter half-life. It may be reasonable to recommence at a prophylactic or intermediate dose and escalate up to a treatment dose if there is no re-bleeding. Insertion of a temporary inferior vena cava (IVC) filter should be considered if early resumption of anticoagulation is unsafe, particularly if the episode of VTE was diagnosed within the previous 4 weeks, although evidence of benefit is lacking.

Factors associated with risk of bleeding in those with VTE are likely similar to those with atrial fibrillation. It should be borne in mind that in patients with previous unprovoked VTE, continuing anticoagulation after the first three months of treatment is associated with a risk of recurrent VTE of about 1.5 per 100 person-years compared to a risk of 10.3 per 100 person-years in the first year after discontinuation of treatment. The risk of major bleeding is about 1.7 per 100 person-years with VKAs and 1.1 per 100 person-years with DOACs. However, the overall

Table 57.2 Factors to consider before re-initiation of anticoagulation following GI bleed or ICH in patients with atrial fibrillation.

Modifiable risk factors for bleeding on anticoagulants	Factors favouring withholding DOACs after GI bleed in patients with AF	Factors favouring withholding DOACs after ICH in patients with AF
Hypertension	Unidentifiable site of bleeding	No reversible cause of bleeding
Concomitant medication – antiplatelet/NSAID/SSRI	Multiple angiodysplasias in GI tract	Multiple cerebral microbleeds
Non-adherence to anticoagulation	No reversible/treatable cause	Severe intracranial bleed
Hazardous occupation/hobbies	Bleeding during treatment interruption	Older age
Choice of anticoagulant	Chronic alcohol abuse	Bleeding during interruption of anticoagulation
Dosing of anticoagulant	Older age	Uncontrolled hypertension
Potentially modifiable:	Careful reassessment of stroke and bleeding risk	Bleed on adequately or under-dosed DOAC
Falls risk		
Anaemia		
Warfarin management		
		Chronic alcohol abuse
		Need for dual antiplatelet therapy after PCI

(*Source:* Adapted from Steffel J *et al.* 2021).

case fatality rate for recurrent VTE is 4% compared to 9% for major bleeding. Where licensed, lower doses of DOACs should be used in long-term treatment.

In patients with mechanical heart valves, resumption of anticoagulation is particularly problematic after intracerebral haemorrhage. In general, resumption of therapeutic anticoagulation should be delayed for 2 weeks to reduce the risk of haemorrhagic complications. However, resumption at day 6 might be considered for patients at high thrombotic risk (mitral or dual valve replacement, cage-ball prosthesis, atrial fibrillation) after taking account of the individual's composite thrombotic and haemorrhagic risks.

The patient with thrombosis

Thrombosis at young age

The overall management of thrombosis at a young age follows similar principles to management in those of older age as discussed in Chapter 49.

In younger populations, thrombophilia testing is more likely to be considered. However, the acute management of lower limb deep vein thrombosis (DVT) and/or pulmonary embolism is generally unaffected by the presence or absence of a laboratory-diagnosed thrombophilic tendency. Moreover, acute phase effects and anticoagulation interfere with coagulation-based assays. Therefore, there is no role for routine thrombophilia testing in the acute setting with the possible exception of solid-phase testing for cardiolipin and β_2-glycoprotein 1 antibodies in selected patients (generally under 50 years) with an episode of venous thrombosis that was unprovoked or provoked by a minor risk factor since, particularly if both are positive, this might suggest a preference for LMWH/warfarin over a DOAC and a need to repeat testing after 12 weeks.

One uncommon but important group is young patients with extensive ilio-femoral DVTs. If left-sided, the May-Thurner syndrome should be considered, i.e. compression of the left common iliac vein by the right common iliac artery, since such patients may be considered for venoplasty and stenting. Some patients, typically in their late teens or twenties, with uni- or bilateral proximal DVTs may have congenital abnormalities of the inferior vena cava and a high risk of recurrent DVT that generally warrants long-term anticoagulation, and vascular surgery input may be considered.

The role of age in the prediction of recurrent venous thrombosis is uncertain as illustrated by its inclusion in different prediction rules for recurrent VTE in those after an unprovoked first event – in the DASH score age $\leq$50 years is regarded as a risk factor, in the HERDOO2 score in women age $\geq$65 years is regarded as a risk factor, and age is not included as a risk factor in the Vienna prediction model.

Haematologists are not infrequently asked about investigation of young patients with arterial thrombotic events. In general, the risk factors that are important in older patients, such as hypertension, diabetes mellitus, dyslipidaemia and cigarette smoking, are also important in younger patients. The finding of a heritable thrombophilia would not alter management and testing for heritable thrombophilias is not recommended. However, testing for antiphospholipid antibodies is recommended when other vascular risk factors are absent as this may influence the choice of antithrombotic therapy.

Thrombosis at unusual sites

Thrombosis at unusual sites is dealt with in Chapter 49.

Recurrent thrombosis or thrombosis despite anticoagulation

The approach to recurrent venous thrombosis depends on whether it occurred after anticoagulation had been discontinued, or was due to sub-therapeutic anticoagulation, or while therapeutically anticoagulated. The mode of anticoagulation is also important. Long-term anticoagulation reduces the relative recurrence risk by 80–90% so occasional recurrences are inevitable. The important first step is senior review of the imaging because the radiological diagnosis of recurrence is often difficult and the potential long-term management implications are significant.

Recurrent thrombosis off anticoagulation generally requires resumption of anticoagulation and lowers the threshold for continuing treatment long-term.

Recurrence may be the result of sub-therapeutic anticoagulation arising from poor compliance or from of factors that result in inappropriate dosing. This is easiest to judge in patients on vitamin K antagonists because of INR measurement, and details should be sought about compliance and interacting drugs and vitamin K intake. A switch to a DOAC might be considered in those with poor INR control because of, for example, difficulties with clinic attendance for INR monitoring. Good compliance is also important in patients on DOACs and LMWH because of their short half-lives. Other possible causes of sub-therapeutic anticoagulation with DOACs are interacting drugs, failure to take therapeutic dose rivaroxaban with food, malabsorption, and possibly obesity with edoxaban and dabigatran where data on use in the obese is lacking.

Prospective studies and randomised trials show a significantly higher risk of VTE recurrence in patients with cancer-associated thrombosis. Antiphospholipid syndrome (APS) is also associated with an increased risk of recurrent thrombosis. Randomised trials have confirmed an increased risk of arterial thrombotic events in those with an initial venous or arterial episode treated with DOACs compared to warfarin, particularly in those who are triple antiphospholipid antibody positive. A falsely increased INR should be excluded in patients

with the antiphospholipid syndrome and recurrent thrombosis on warfarin whose INR is apparently within the therapeutic range. This can be done by using an alternative thromboplastin that is insensitive to the lupus anticoagulant or by a chromogenic factor X assay where an expected level would be 20–40% if the INR is 2.0–3.0. If the INR has been genuinely within the therapeutic range, the target INR should be increased from 2.5 to 3.5, potentially after a period of single agent therapeutic dose LMWH. Alternatively, aspirin and/or hydroxychloroquine could be added. For those with recurrent thrombosis at an INR of 3.0–4.0, possible approaches include the addition of aspirin, a switch to LMWH or fondaparinux, or the adjunctive use of immunosuppressive or immunomodulatory agents such as rituximab, complement inhibitors or mammalian target of rapamycin (mTOR) inhibitors.

In those without APS who have a recurrent thrombotic event on warfarin and a therapeutic INR, the target INR should be increased from 2.5 to 3.5 after a period of LMWH. Aspirin could be added if the recurrence is despite anticoagulation with an INR of 3.0–4.0. If INR control is poor and a DOAC is contraindicated, consideration could be given to long-term parenteral therapy with LMWH or fondaparinux.

Causes of recurrent thrombosis while on therapeutic anticoagulation are listed in Table 57.3.

Thrombosis in presence of bleeding risk or thrombocytopenia

The combination of thrombosis and thrombocytopenia is challenging to manage because of competing thrombotic and bleeding risks. There are a number of causes with cancer being the most frequently encountered, particularly in those with haematological malignancies and/or receiving chemotherapy. Other well-recognised causes include sepsis, cirrhosis, APS, heparin-induced thrombocytopenia (HIT), ITP, DIC, paroxysmal nocturnal haemoglobinuria (PNH) and vaccine-induced immune thrombocytopenia and thrombosis (VITT). The microangiopathies, such as TTP, haemolytic uraemic syndrome (HUS) and HELLP syndrome, usually cause thrombosis limited to small vessels. In view of the disparate nature of these conditions, accurate diagnosis is essential including, when thrombocytopenia is new or unexpected, exclusion of pseudothrombocytopenia with a blood film and citrated FBC. It should be borne in mind that pseudothrombocytopenia in citrated blood can occur during treatment with glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists.

Table 57.3 Causes of recurrent thrombosis while on therapeutic anticoagulation.

Cancer
Myeloproliferative neoplasm with high cell counts
Antiphospholipid syndrome especially if on DOAC treatment and triple-positive antiphospholipid antibodies
Heparin-induced thrombocytopenia
Paroxysmal nocturnal haemoglobinuria
Behçet's syndrome
Antithrombin deficiency (heparin)
Vascular abnormalities – May-Thurner syndrome, thoracic outlet syndrome, congenital abnormalities of the inferior vena cava
Inappropriate choice or dosing of anticoagulant
Poor adherence

In patients with cancer and thrombocytopenia, LMWH is generally the preferred therapeutic option. Guidelines suggest full treatment doses can be safely administered down to a platelet count of 50×10^9/L. In the setting of acute venous thrombosis (within 30 days of the index event), platelet support should be given when necessary to elevate the platelet count to $>50 \times 10^9$/L. If the platelet count cannot be elevated, a 50% treatment dose can be given if the platelet count is $25–50 \times 10^9$/L. Anticoagulation is generally contraindicated if the platelet count is $<25 \times 10^9$/L and insertion of a retrievable IVC filter might then be considered in the setting of acute venous thrombosis and refractory thrombocytopenia. Full-dose anticoagulation should be resumed as the platelet count recovers to $>50 \times 10^9$/L. In those with platelet counts of $25–50 \times 10^9$/L and lower risk acute thrombotic events, such as distal venous thrombosis or subsegmental pulmonary emboli, or those whose VTE event was >30 days earlier, a 50% treatment dose or a prophylactic dose without platelet support are suggested.

In patients with acute lymphoblastic leukaemia receiving asparaginase, International Society on Thrombosis and Haemostasis guidance suggests weekly monitoring of antithrombin levels. If venous thrombosis occurs, LMWH is suggested in preference to a DOAC if a platelet count $<50 \times 10^9$/L is anticipated. Anti-Xa levels should be monitored if plasma antithrombin activity is decreased below 50–60%. For life-threatening VTE, such as cerebral venous sinus thrombosis (CVST) or central pulmonary embolism, concurrent administration of antithrombin concentrate is suggested until therapeutic anticoagulation and clinical stability are established.

In patients with other causes of thrombosis and thrombocytopenia, management depends on the underlying condition.

Thrombosis and cancer

Up to 20% of all cases of VTE occur in patients with cancer and thrombosis is one of the leading causes of death in cancer patients after cancer itself. The 12-month cumulative incidence of VTE after a cancer diagnosis is >3%, the risk varying between cancer sites and increasing as cancer progresses. Outcomes, including recurrent thrombosis, bleeding and mortality, of patients with cancer and thrombosis are worse than the outcomes of either condition on its own. The reasons for the association of cancer and thrombosis are multifactorial and include

the prothrombotic effects of cancer and its treatments, reduced mobility, hospital admission, central lines and surgery.

For two decades, LMWH for 6 months has been the standard treatment of VTE in patients with cancer-associated venous thrombosis because of trial data showing a reduced recurrence risk compared to warfarin without an increased risk of major bleeding. The risks and benefits of continuing anticoagulation should be reviewed at 6 months. Data beyond this are sparse but generally anticoagulation is continued with regular review in those with active cancer, taking account of bleeding risks and patient status and preferences. Recent trial evidence suggests that the direct oral factor Xa inhibitors are as effective and safe as LMWH in the initial treatment of cancer-associated venous thrombosis. Recent national and international guidelines support their use as first-line therapy although LMWH is preferred in those with a high risk of bleeding including those with luminal gastrointestinal cancers with an intact primary, patients at increased risk of bleeding from the genitourinary tract, patients with active gastrointestinal mucosal abnormalities or patients taking potent inhibitors or inducers of P-glycoprotein and/or cytochrome P450 3A4. The increased risk of bleeding from gastrointestinal cancers that was seen in the trials with edoxaban and rivaroxaban was not observed with apixaban. Current guidelines support anticoagulation of incidental venous thrombosis, at least for 6 months.

The risk of recurrent on-treatment thrombosis is increased in patients with cancer-associated venous thrombosis. Non-compliance with treatment should be excluded and a possible diagnosis of HIT should be considered but is unlikely. There are no randomised trials to inform management. Current recommendations are to switch to LMWH if being managed on other anticoagulants, to increase the dose to therapeutic if being managed on a sub-therapeutic dose of LMWH, and to increase the dose by about 25% if currently on a therapeutic dose of LMWH. With enoxaparin the once-daily therapeutic dose regimen may be switched to the twice daily dose.

In other settings, thrombosis occurs together with a significant bleeding concern. For example, VTE is common in patients with primary brain tumours or cerebral metastases. Such patients were largely excluded from randomised trials of anticoagulants and management is complicated by the high rate of spontaneous ICH, particularly in certain tumour types such as non-small cell lung cancer or renal cell cancer. Retrospective data suggest that LMWH does not increase the risk of ICH in those with cerebral metastases but may increase the risk in those with primary brain tumours. Preliminary observational data in patients with primary brain tumours suggest that DOACs may be associated with a lower risk of ICH but LMWH is generally preferred initially because of the shorter half-life. In view of the paucity of data, the management of patients with intracranial lesions and acute VTE should be discussed with a multidisciplinary team including neurosurgeons and oncologists, and treatment decisions individualised.

Bleeding or thrombosis and systemic disease

General effects of inflammation

The underlying mechanisms whereby haemostasis is disturbed in patients with inflammation are outlined in Chapter 43.

Inflammation is such a common feature in patients with thrombosis that in general management is unaffected unless a decompensated state has been reached and there is an associated bleeding tendency as in DIC. The most widely used anticoagulants are LMWH and DOACs and an inflammatory state does not usually alter their efficacy. However, problems may arise in patients in whom intravenous unfractionated heparin is preferred because of its reversibility. In such patients, in particular, the phenomenon of heparin resistance may arise. This is characterised by difficulty in achieving the target APTT ratio using standard doses of heparin. The causes are multifactorial and include elevated acute phase proteins and activated cell surfaces to which an increasing proportion of long-chain heparin molecules non-specifically bind, increased factor VIII and fibrinogen levels that shorten the baseline APTT and therefore result in an increased heparin requirement to prolong the ratio into the therapeutic range, and acquired antithrombin deficiency. Management of this problem can be difficult and it is thought that measurement of anti-Xa levels (typical therapeutic range 0.3–0.7 IU/mL by chromogenic assay) may be superior to APTT monitoring. In patients with acquired antithrombin deficiency and without a history of thrombosis, a role for antithrombin replacement has not been demonstrated outside the setting of cardiac surgery.

Diagnosis and management of DIC are outlined in Chapter 43.

Liver disease

Liver disease is dealt with in Chapter 43.

Amyloidosis

Amyloidosis is dealt with in Chapter 43.

Behçet's syndrome

Behçet's syndrome is an inflammatory multiorgan vasculitis characterised by recurrent oral and genital ulcers, and involvement of the skin, eyes, joints, gut and central nervous system. The overall prevalence is 10 per 100,000 inhabitants but this varies considerably geographically with a prevalence of over 100 per 100,000 in Turkey and 3 per 100,000 in European populations. It affects men and women equally but young men under 30 years have a greater likelihood of a more severe course. Its onset is rare after 40 years.

Behçet's syndrome is unique amongst vasculitides because it predominantly affects veins and probably causes inflammation and activation of the vein wall endothelium as well as activation of neutrophils and platelets. Vascular involvement is usually in the form of thrombosis or aneurysm formation and is present in up to 40% of cases, with 75% of these being venous. Lower extremity venous thrombosis is the most common manifestation although vena cava thrombosis, pulmonary artery aneurysms, cerebral venous sinus thrombosis, Budd-Chiari syndrome, and peripheral artery and abdominal artery aneurysms also occur. The recurrence risk of lower extremity venous thrombosis is high with one study reporting a 45% recurrence risk at 2 years.

Endothelial activation is thought to induce thrombosis through reduced nitric oxide production and increased plasma levels of von Willebrand factor and P-selectin. Fibrinogen structure and function may be altered by reactive oxygen species produced by neutrophil activation and fibrin clots may be less susceptible to plasmin-mediated lysis. Anti-endothelial and antiphospholipid antibodies are seen in some patients. Imaging studies show thickened vessel walls in patients with vascular Behçet's syndrome compared to controls, even in non-thrombotic areas.

Arterial lesions may be occlusive or aneurysmal. The latter results from vasculitic destruction of the media, arterial dilatation, and fibrosis with inflammatory destruction of the vasa vasorum.

European Alliance of Associations for Rheumatology (EULAR) guidelines recommend glucocorticoids and immunosuppressives such as azathioprine, cyclophosphamide or cyclosporine A as the primary therapy for acute venous thrombosis. Other immune therapies are being investigated. The role of anticoagulants is uncertain and there are concerns about their use in patients with associated pulmonary artery aneurysms because of potential bleeding risks. Mechanical thrombectomy should be avoided as it may predispose to aneurysm formation. In view of the uncertainties, it is suggested that patients with significant new thrombotic events should be discussed with an expert in one of the three UK Behçet's syndrome centres (London, Birmingham, Liverpool).

Sickle cell disease

Sickle cell disease (SCD) is associated with an acquired prothrombotic state. This is most evident in the early onset of arterial complications such as stroke in children. It has been observed in epidemiological studies that reported, for example, a 10–15% cumulative incidence of VTE by the age of 40 years and a fivefold increase in the risk of VTE in pregnancy compared to women without SCD. The recurrence risk of VTE in SCD is also high at over 35% at 5 years. The risk of pulmonary embolism appears to be higher than for DVT and this may be partly attributable to *in situ* pulmonary thrombosis. Mortality appears to be higher in people with SCD and a history of VTE compared to those without VTE. Risk factors for VTE in people with SCD include frequent hospital admissions, exposure to erythropoietin or blood transfusions, insertion of central venous catheters and surgical splenectomy.

The molecular and cellular changes in SCD result in two primary pathologies: vaso-occlusion-mediated ischaemia/reperfusion and increased circulating levels of free haem caused by chronic haemolytic anaemia. The former leads to free radical-mediated oxidative stress and increased vascular inflammation. The latter results in depletion of haptoglobin and haemopexin, which scavenge free haemoglobin and free haem respectively, and this leads to reduced nitric acid bioavailability. This in turn causes platelet activation as well as activation of endothelial cells and neutrophils. Monocyte activation leads to increased production of inflammatory cytokines that can induce tissue factor expression and thereby initiate coagulation. Further thrombin generation may result from activation of the contact system through, for example, formation of neutrophil extracellular traps (NETs). Sickle red cells themselves have numerous receptors that interact with the vessel wall and result in post-capillary venule occlusion, and increased numbers of phosphatidylserine-positive sickle red cell-derived extracellular vesicles have been associated with markers of thrombin generation. The coagulation system is activated with increased markers of thrombin generation and fibrin turnover. Thus the prothrombotic tendency of sickle cell disease reflects an underlying thrombo-inflammatory state with numerous factors altering the haemostatic balance.

The cumulative effect of stasis, altered rheology, vascular fibrin deposition and chronic inflammation leads to a chronic vasculopathy and multi-organ damage. Those affected by deep vein thrombosis and/or pulmonary embolism may develop long-term complications such as the post-thrombotic syndrome and chronic thromboembolic pulmonary hypertension.

In general, prospective trials of anticoagulation have not included people with SCD and management of VTE follows guidelines developed for the general population. In view of the high risk of recurrent VTE that has been reported, it seems reasonable to consider long-term anticoagulation after a first event even in those with less severe disease or where the incident event was related to hospitalisation. Based on few data, DOACs seem a reasonable option at this point. However, before embarking on long-term anticoagulation, it is important to consider the underlying risk of bleeding such as from intracranial haemorrhage due to central nervous system vasculopathy as well as other comorbidities associated with an increased bleeding risk and to take account of patient preferences.

Paroxysmal nocturnal haemoglobinuria

Thrombosis is a frequent and feared complication of PNH with historically 29–44% of patients having at least one thrombotic

event during follow-up. The majority of thrombotic episodes (75%) are venous with over 50% of these involving either the hepatic or portal veins. Although more common in those with large PNH clones (>50% PNH granulocytes), the risk is also increased in those with smaller clones compared to the background population.

The pathogenesis of thrombosis in PNH is multifactorial and the efficacy of complement inhibitors in preventing thrombotic events illustrates the central importance of complement activation. Although not fully characterised, it is thought that two main mechanisms are responsible – haemolysis-related and non-haemolysis-related. Complement-mediated destruction of red cells with intravascular haemolysis results in the release of danger-associated molecular patterns (DAMPs), such as cell-free haemoglobin and cell-free haem, into plasma. These have multiple pro-thrombotic effects with activation of platelets, neutrophils, monocytes, endothelial cells and coagulation factors. Haemolysed red cells release phosphatidylserine microvesicles that can support thrombin generation. Recognition that thrombotic episodes occur in patients with low levels of haemolysis suggest that platelet, white cell and endothelial factors likely play a pivotal role. There are multiple bidirectional interactions between the complement and coagulation systems leading to their reciprocal activation and thrombin generation.

In patients who present with a thrombotic event, the initial step is anticoagulation. This is standard with low-molecular-weight or unfractionated heparin followed by warfarin. The DOACs have not been evaluated in PNH although they would seem reasonable options in individual patients. However, the key intervention, ideally within 24 hours, is with a C5 inhibitor such as eculizumab or ravulizumab which prevents thrombosis progression in the acute phase and appears particularly important in the Budd-Chiari syndrome. These agents are also required long-term because anticoagulation alone is insufficient for secondary prevention. There is uncertainty over the duration of anticoagulation in patients on complement inhibitors whose haemolysis is well controlled and who may have bleeding risks due, for example, to thrombocytopenia, and advice in individual cases should be sought from an expert in one of the two UK centres, London and Leeds.

Myeloproliferative Neoplasms (MPN)

Thrombosis

In Philadelphia-negative MPNs, the pooled prevalence of thrombosis at the time of diagnosis is 20%. Arterial events are more common in older patients and venous thrombosis is more common in younger patients. In females thrombosis is more commonly seen in unusual sites such as splanchnic veins, cerebral venous sinuses and in the placenta. During long-term follow-up the increased risk of an arterial thrombotic event is of a similar degree to cigarette smoking and the risk of venous thrombosis is increased to a similar degree as heterozygosity for factor V Leiden. Cardiovascular risk factors in the general population likely also contribute to the thrombotic risks of MPNs. The risk of thrombosis is higher in those over 60 years and/or with a past history of thrombosis and/or who carry the *JAK2V617F* mutation.

There are no randomised trials to inform anticoagulant management following an episode of venous thrombosis. Current management in polycythemia vera involves phlebotomy to a haematocrit of <45% in both males and females and the addition of hydroxyurea or interferon and anticoagulation. Retrospective data suggest that DOACs are as safe and effective as warfarin. Following an arterial event antiplatelet therapy is used in place of anticoagulation and some recommend twice daily low-dose aspirin because of increased platelet turnover. A similar approach following venous and arterial thrombotic events but without phlebotomy is generally taken with essential thrombocythemia. Some authorities add aspirin to anticoagulation in those with venous thrombosis and cardiovascular risk factors but the bleeding risk is increased.

Bleeding

Bleeding is less common than thrombosis in patients with MPNs and mostly involves gastrointestinal, mucosal and cutaneous bleeding. The cause is multifactorial and may of course result from thrombocytopenia or antithrombotic therapy. The most widely accepted mechanism, however, is acquired von Willebrand disease. Although associated with extreme thrombocytosis, evidence for acquired von Willebrand disease is found in over 50% of patients with platelet counts $<1000 \times 10^9$/L. Laboratory diagnosis can be difficult outside a specialist centre. Management is supportive, antithrombotic therapy may require interruption and cytoreduction to normalise the platelet count.

Managing anticoagulation for surgery and procedures

Advice is largely based on the British Society for Haematology guidelines on the peri-operative management of anticoagulation and antiplatelet therapy. Management entails assessment of the procedure-related bleeding risk and potential consequences of bleeding if anticoagulation continues on the one hand and the thrombotic risk and consequences if anticoagulation is interrupted. The assessment also needs to take into account patient-specific clinical factors and of the pharmacological properties of different anticoagulants.

Some procedures such as much dental work, cataract surgery, pacemaker insertion, joint injections and certain endoscopic procedures are of low bleeding risk and do not require anticoagulation to be interrupted. In general, however, the

British Society for Haematology guidelines suggest that the surgeon, dentist or interventional radiologist has to assess the bleeding risk for the individual patient and discuss this and the plan for peri-procedural anticoagulation with them, recording the plan clearly in the notes, including for when the patient is discharged.

Warfarin and other vitamin K antagonists

Warfarin has a half-life of about 36 hours and as its effect subsides γ-carboxylated vitamin K-dependent clotting factors are fully synthesised and secreted by hepatocytes. Therefore, with a starting INR in the therapeutic range a week beforehand, warfarin generally needs to be stopped 5 days before elective surgery to enable the INR to normalise. Ideally, the INR should be rechecked the day before surgery to allow vitamin K to be administered if the INR is still ≥1.5, thereby reducing the need for cancellation. The INR should also be checked on the day of surgery.

Patients who are judged at high risk of thrombosis when VKA treatment is interrupted should be considered for 'bridging', i.e. the administration of therapeutic dose LMWH or UFH during the period of sub-therapeutic oral anticoagulation. LMWH has the advantage over UFH of ease of use with fixed weight-based dosing and outpatient administration. Generally, the heparin is commenced 48 hours after the last dose of warfarin. LMWH is discontinued at least 24 hours and intravenous UFH 4–6 hours before the procedure. Following high-bleeding risk surgery, patients should generally not recommence therapeutic dose of heparin until at least 48 hours after the procedure although thromboprophylactic doses should be given if indicated. Because of its slow onset of action, warfarin can generally be resumed the evening of or the day after surgery but in circumstances where LMWH is more appropriate in the short term, it may be reasonable to delay warfarin's reintroduction. The approach is outlined in Table 57.4.

Settings where bridging doses of heparin might be considered when warfarin is interrupted are shown in Table 57.4.

Although this reflects current practice, it should be acknowledged that evidence showing the benefit of bridging is lacking while increased bleeding risks have been observed in some studies.

Additionally, patients with an episode of VTE within the previous month who require interruption of anticoagulation for surgery that cannot be delayed should be considered for insertion of a retrievable IVC filter until anticoagulation can be safely resumed post-operatively.

In patients requiring emergency surgery who can wait for 6–8 hours, 5 mg intravenous vitamin K can correct the INR. If a delay is not possible, PCC 25–50 IU/kg can be given. Post-operative management is similar to that following elective surgery.

Table 57.4 Settings where bridging doses of heparin might be considered when warfarin is interrupted.

	Consider **bridging with treatment dose heparin in**
VTE	Patients with a VTE within previous 3 months. Very high-risk patients such as patients with a previous VTE whilst on therapeutic anticoagulation who now have a target INR of 35.)
AF	Patients with a previous stroke/TIA in last 3 months. Patients with a previous stroke/TIA and three or more of the following risk factors: • Congestive cardiac failure • Hypertension (>140/90 mmHg or on medication) • Age >75 yrs • Diabetes mellitus
MHV	MHV patients other than those with a bileaflet aortic valve and no other risk factors

AF, atrial fibrillation; INR, International Normalised Ratio; MHV, mechanical heart valve; TIA, transient ischaemic attack; VTE, venous thromboembolism.
(*Source:* Keeling D *et al.* 2016/John Wiley & Sons).

Direct oral anticoagulants

Similar general principles apply to DOACs as to VKAs in considering interruption of anticoagulant therapy before and after elective surgery. However, the shorter half-life of the DOACs means that bridging with heparin is generally not recommended and appears to increase the bleeding risk without reducing the risk of thrombotic events. Again, plans should be discussed in advance with the patient and be clearly recorded in the notes.

As for warfarin, some procedures carry no significant bleeding risk and do not require interruption of anticoagulant therapy. In practice, for such minor procedures, it may be reasonable to schedule the intervention 18–24 hours after the last DOAC intake and restart 6 hours later, thereby skipping one dose of (twice daily) apixaban or dabigatran or timing the intervention at the trough level of (once daily) edoxaban or rivaroxaban.

For procedures associated with high or low bleeding risks, the recommended times to discontinue DOACs in advance are shown in Table 57.5. In patients whose creatinine clearance is <30 mL/min, the interval between the last DOAC dose and elective surgical intervention may need to be extended.

Following low bleeding risk procedures, DOACs can generally resume 12–24 hours later if haemostasis has been fully secured. In those with a high post-procedural bleeding risk, reintroduction of the full dose of the DOAC should be delayed for at least 48 hours. Daily prophylactic heparin should be considered in patients at high risk of venous thrombosis prior to DOAC recommencement.

Table 57.5 DOAC perioperative management scheme.

DOAC	Procedural bleeding risk	Perioperative DOAC management									
		Day −5	Day −4	Day −3	Day −2	Day −1	Day 0	Day +1	Day +2	Day +3	Day +4
Direct Xa Inhibitors *and* Dabigatran (CrCl ≥ 50 mL/min)	Low	✓	✓	✓	✓	OMIT	Day of procedure Do not administer any DOAC	✓	✓	✓	✓
	High	✓	✓	✓	OMIT	OMIT		OMIT	Resume day +2 or day +3		✓
Dabigatran (CrCl < 50 mL/min)	Low	✓	✓	✓	OMIT	OMIT		✓	✓	✓	✓
	High	✓	OMIT	OMIT	OMIT	OMIT		OMIT	Resume day +2 or day +3		✓

✓ – DOAC may be taken or administered.
(*Source:* Saja K, 2022/John Wiley & Sons).

When emergency surgery is required, discussion with the surgeon and anaesthetist is necessary to assess the risks and timing of the surgical intervention. Knowledge of the specific DOAC the patient is taking, the timing of the last dose, and the current renal function are of considerable importance in estimating the potential degree of anticoagulation. Other factors such as weight and interacting drugs are generally of less significance. A normal clotting screen (PT and APTT) does not exclude the presence of a significant concentration of a direct oral Xa inhibitor, although a normal thrombin time effectively excludes the presence of dabigatran. Where available, rapid access to individual DOAC concentrations can be helpful in assessing the degree of anticoagulation and the likely timeframe for waning of the effect, in turn assisting with decision-making over the timing of surgery. Where possible, surgery associated with a bleeding risk should be postponed in patients in whom a significant anticoagulant effect is known or is likely, taking into account the half-life of the DOAC and the risks of delaying surgery.

In patients on dabigatran where surgery or urgent procedures cannot be delayed and carry a bleeding risk, idarucizumab is licensed for rapid reversal of its anticoagulant effects. The dose is as for reversal of dabigatran for life-threatening or uncontrolled bleeding. Andexanet alfa has not been evaluated for its efficacy in reversing the direct oral Xa inhibitors prior to emergency surgery and is not licensed for this indication. In clinical practice where surgery cannot be delayed, PCC has been used but it should be appreciated by clinicians that it is not an antidote and its efficacy in this setting is uncertain. British Society for Haematology guidelines suggest that a pragmatic approach might be to proceed with surgery considering PCC only in the event of diffuse coagulopathic bleeding. Tranexamic acid may also be of benefit and should be given.

General notes in consultative haemostasis and thrombosis

Balancing risks and benefits

When practising consultative haemostasis, it is often true that decisions may need to be compromised and represent the best balance between competing risks. In nearly all cases, the intention is to minimise the overall risk and maximise the benefit for the patient. Usually, this means balancing the risks (both the immediate risk and the consequences of unwanted events) of thrombosis against those of bleeding. It is often not easy to compare risks of bleeding with risks of thrombosis. Thrombosis may also have longer, indirect effect such as pulmonary hypertension or postphlebitic syndrome and critical site bleeding may also have long-term consequences. For longer-term decisions, it is important to consider the patient's quality of life and how burdensome a theoretically good solution might be. Many patients intensely dislike taking medication and for others, the shock of a thromboembolic event was so traumatic that any risk of bleeding from anticoagulation is outweighed by the feeling of safety and reassurance it confers.

Making recommendations

Problems in hospital management can arise if advice is not communicated clearly or is too tentative. It is important that unambiguous instructions are given that deal with likely alternative developments. Obviously, these need to be communicated clearly to the consulting team and written in the patient's notes. For surgical management a clear and complete plan should be placed in the patient notes with relevant contact details. It is dangerous to assume that any aspect of management is obvious or self-explanatory.

Genetic consultations

Determining the precise cause of a haemorrhagic or thrombotic tendency often leads to genetic testing. This is increasingly readily available and increasingly useful as more variants are identified and their phenotypic correlates described. In the UK, gene panels for both thrombotic and bleeding disorders, including thrombocytopenia are available via the NHS genomics network. This can be very helpful in predicting the phenotype – for example identifying heparin-binding variants of antithrombin which have a lower thrombotic risk than other forms of deficiency. It may also help interpretation of laboratory results and for example, define the type of VWD and predict the response to therapy. Some guidelines recommend genetic testing over laboratory tests where the latter are poorly standardised or difficult such as for type 2B VWD and platelet-type VWD. However genetic testing rarely identifies an abnormality when a clinical phenotype is not apparent clinically or on laboratory testing.

The largest advances have been where there is a clinical phenotype but no diagnostic test – a good example is thrombocytopenia. Where many possible genetic abnormalities may be responsible then genetic panels can be set up and hundreds of genes analysed at the same time. Multiple genetic variants can be combined to give a propensity score for thrombocytopenia. However, the analysis may reveal genetic variants which have implications beyond those expected. For example, some genetic variants responsible for thrombocytopenia, such as *ACTN1* have no phenotype beyond the low platelet count whereas others, such as *ANKRD26* have a clear association with leukaemia. The risk of leukaemia may be unexpected and unwelcome information for the patient and their family with a significant impact on their lives. At the same time, the doctor in possession of the information has an obligation to pass this on. It is therefore essential to fully explain and counsel patients of all the possible outcomes before obtaining their consent for genetic tests. Another problem for the future is that variants identified today without any known clinical significance may later be found to have important implications and the patient may expect to be updated on the interpretation of their genetic status as information changes. This will be a major task for genetic services.

Genetic testing is also available for family studies where a clear hereditary cause is already identified such as haemophilia or VWD. This is useful for assessing risk of transmission and can be used for antenatal diagnosis either for termination or to plan delivery. Genetic testing is not usually justified in children below the age of competence for consent and should be avoided unless it will have an important effect on management. Phenotypic testing is usually sufficient to direct management.

End-of-life decisions

Thrombosis and haemostasis both increase significantly with age. Moreover, not only are they frequent complications of many other disorders, the therapy, particularly anticoagulants, may have significant interactions with other treatments and may be burdensome to administer. Finding the best balance of risk and benefit without making life miserable due to repeated injections or having to spend a lot of time in or attending hospital becomes a particularly acute and difficult problem towards the end of life. The advent of new anticoagulants have simplified management as have near-patient testing devices and home visits. It is important not to 'strive officiously' and to remember that quality of life is particularly important when there is only little left.

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Hoffbrand's Postgraduate Haematology, Eighth Edition. Edited by Adam J Mead, Michael A Laffan, Graham P Collins, Deborah Hay, and A Victor Hoffbrand (Consulting Editor).

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Companion website: www.wiley.com/go/mead/HPH8e

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